

THE DYNAMIC RELATIONSHIP BETWEEN LANGERHANS CELLS AND INTRAEPIDERMAL
NERVE FIBERS IN THE MOUSE AND RAT FOOTPAD

By

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Submitted to the graduate degree program in Molecular and Integrative Physiology and the
Graduate Faculty of the University of Kansas in partial fulfillment of the requirements for the
degree of Doctor of Philosophy.

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Abstract

Skin disorders are often associated with immune and nervous system dysfunction. Intraepidermal nerve fibers (IENFs) detect mechanical, thermal, and noxious stimuli. Although immune cells such as mast and T cells can alter IENFs, it is unclear whether Langerhans cells (LCs), the resident antigen-presenting cell of the skin, do so as well. These studies examine the relationship between IENFs and LCs.

LCs are intimately associated with IENFs, and *in vitro* studies suggest they possess neurotrophic properties. The objective of the first study was to determine if LCs regulate IENFs in skin by ablating LCs using diphtheria toxin in Lang-DTR mice, a strain genetically engineered to express the diphtheria toxin receptor selectively on LCs. In 1 month old mice, LC depletion resulted in 31% and 43% decreases in IENFs immunoreactive (ir) for the pan-neuronal marker PGP9.5 and calcitonin gene-related peptide (CGRP). CGRP-negative (non-peptidergic) IENFs were unchanged. Therefore, LCs are necessary for maintaining CGRP-ir IENFs. Since IENFs are modulated by neurotrophic factors, we determined if LC depletion results in reduced epidermal neurotrophin gene expression. LC depletion decreased NGF and GDNF gene expression by roughly 75% and 90%, respectively whereas BDNF did not change. Thus, LCs are likely to play a substantial role in epidermal NGF and GDNF production, and loss of CGRP-ir IENFs after LC depletion is likely due to diminished trophic support. Behavioral testing was performed to determine if LC depletion alters thermal and mechanical sensitivity. LC depletion increased mechanical sensitivity but did not affect thermal sensitivity. Therefore, LCs play an essential role in maintaining CGRP-ir IENFs and determining mechanical sensitivity in 1 month old mice.

Because age can alter LCs and IENFs, we examined LCs and IENFs in 10 month old control and LC-depleted mice. LC and PGP9.5-ir IENF numbers were reduced in older mice. LC

depletion further reduced PGP9.5-ir IENF density by 52%. Numbers of CGRP-ir IENFs did not change, but LC depletion reduced non-peptidergic IENFs by 98%. This implies that LCs exert age-dependent influences on different IENF subtypes, changing from CGRP-ir to non-peptidergic with age. LC depletion did not alter NGF, GDNF or BDNF gene expression in older mice. LCs therefore play a major, age dependent role in determining cutaneous innervation density, neurotrophic factor expression, and mechanical sensitivity.

Neuro-immune cell interactions are altered in individuals with diabetes, which may contribute to cutaneous complications. The objective of the second study was to determine if the relationship between LCs and IENFs is altered in the rat with streptozotocin-induced diabetes. Footpad LC density was reduced after 4 weeks of diabetes, and LC density and size were reduced at 16 weeks. We examined if age influenced LCs and IENFs and found the proportion of skin occupied by LCs was increased by 141%, while IENFs were reduced by 37% in older rats. The increase in cutaneous LCs with age may have occurred in response to IENF reductions. PGP9.5-ir IENFs were not affected by diabetes in our rats; however, reductions in LCs apparently preceded nerve reductions reported by others at later times. Overall, this study shows that diabetes reduces LCs, which may lead to decreased immunosurveillance and increased susceptibility to infections, as well as reduced innervation in the later stages of diabetes.

Collectively, these data show that LCs are essential in maintaining cutaneous innervation. They appear to play a major, previously unidentified role in determining cutaneous neurotrophin production, shedding light on a new function for these classical 'immune' cells. Their importance is underscored by the finding that eliminating LCs substantially reduces cutaneous innervation. Moreover, this is functionally significant given that mice showed pronounced mechanical hypersensitivity after LC depletion. Interestingly, this nerve loss and

allodynia are quite reminiscent of the clinical picture in many types of peripheral neuropathies. Further, the findings that LCs are altered by both diabetes and aging is consistent with the idea that factors that contribute to neuropathies also alter LCs. Clearly, additional study is warranted to further define the roles of these neuro-immune cells in regulating peripheral innervation.

Dedication

This work is dedicated to my parents Willie and Deborah Doss and my brother Lazarus.

We have come this far by faith.

Acknowledgments

First, I owe all of my life, experiences, and knowledge to God. Without his love, I would not be able to place these words upon this paper. Most of my friends and colleagues always heard me refer to this day as the day when God decides: “Whenever that day that God decides I should graduate, I’ll graduate...” Well, he decided and boy has it been a journey.

To begin with those closest to me, I would like to thank my grandparents, AJ and Roberta Candler. Their love, generosity, and faith are still with me and have brought me through many rough patches. I can only hope they know that I became the first person in our family to receive a Ph.D. Next, I must acknowledge my parents Willie and Deborah Doss. They are my rock and have always been there for me. Whether it was driving across state for a sprained ankle, listening to me rant or ramble at three in the morning, or working 12hr shifts to make sure I had what we needed, you were and are there for me. I am so grateful for what you envisioned for me. You showed me that science and math can be fun. I can still remember as a child we watched NOVA or national geographic around dinner time. So many questions would come into my mind. You encouraged me to find the answers and continue to ask questions. I truly believe that if you didn’t expose me to the many different aspects of life, I would not be where I am. There are not enough words in the world that can describe my appreciation and love for you. Lazarus, my brother, what can I say? You are so strong. I have always looked up to you. Thank you for reminding me that sometimes I need to relax and enjoy life. You truly are a big brother and I love you.

If it wasn’t for my friends Elizabeth Bostic and Adrian Sherman and the rest of the LU crew, I wouldn’t be probably as sane as I am now. When my family was not available, you were there for me. Over the past 10 years, we had so many conversations about our lives and personal journeys to creating a successful career. Thank you. As for KUMC, I am so grateful for

the IGPBS crew: Laurie Shannon, Rachel Williams, Crystal Bethel-Brown, and Mary Shawgo. Laurie, Rachel, and Crystal, you guys have been awesome! All of you have made an imprint in my life and are some of the strongest and smartest women I know. Thank you for being there for me. Mary, I had to say thank you separately because you helped me our first year when I didn't understand what I was doing wrong. I knew the information but couldn't express it. Whether you knew it or not, you gave me the first step to communicating science at a graduate level. I am so appreciative of the time I got to spend with all the IGPBS crew.

Doctor...Doctor....Doctor...Doctor...

I would like to thank the Smithlab past and present: Dr. Arunadah Chakorabarty, Dr. Sarah Tague, Dr. Zaiohui Liao, Dr. Gwenaelle Clarke, Eva Selfridge, Dr. Wohaib Hasan, Tim Donahue, Aritra Bhattacharjee, Elza Kharyatan, and Dr. Dora Krizan-Agbas. When I came into the lab for the first time, I just knew I was going to have some awesome experiences and grow as a person and scientist. Thank you for your assistance through my journey. Especially, Tim, who always had a new story to tell, Aritra, who was my philosopher in the lab, and Elza, who just knew me without me having to explain. Lastly, Dora, you helped me become confident in discussing my views about science. I want you to know that you played a significant role in my development as a scientist. As long I can remember, I will always remember the lessons I learned from and with you. Thank you.

Langston University played a huge role in my journey. Sonya J. Williams, Ph.D., John Coleman, Ph.D., Rosemary Harkins, Ph.D., and Zola Drain, Ph.D. were my mentors at Langston. It wasn't until now that I understand that they groomed me to be a scientist. It was the most fortunate time. With the use of biomedical grants that emphasized exposing young minority students to research, I, along with others, was able to learn so much about science and that I could also contribute to the field. I now understand. Thank you.

In addition to acknowledging my mentors at Langston, I would like to give my appreciation to my committee members: Dr. Paige Geiger, Dr. Kenneth McCarson, Dr. Irina Smirnova, and Dr. Doug Wright. Over the years, I have observed you and learned about science, collaboration between laboratories and researchers from other organizations. I truly am grateful for all of you taking time to help me become a better scientist.

I would like to say a special thank you to Dr. Paul Cheney and Dr. Thomas Imig. When it was time for me to choose a department to join, I did not have the best grades. However, the two of you made me feel at home in the department of Molecular and Integrative Physiology and I appreciate that so much. With respect to the department, I would like to say thank you to the department's Graduate Program Director, Dr. Lane Christenson, and the administrative staff. Thank you for all of your assistance. I also thank the Kansas Intellectual Developmental Disabilities Research Center's administrative and core staff. They made life so much easier.

Finally, I would like to acknowledge my mentor, Dr. Peter G. Smith. When I came to Dr. Smith, I did not have the best grades and was worried about my future. I had no previous graduate experience. Yet, he saw that I could be a scientist. He had faith in me. I am so grateful for you taking a risk and letting me join your lab. I have learned a vast amount of information about science, time management, writing, networking, and the whole grant process. I am so glad that I chose your lab. Thank you.

This work was supported by NIH grants NINDS 1RO1 NS053796, KIDDRRC P30 HD02528, and NIDDK 1F31 DK083845.

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Chapter 1: General Introduction

Nerve-immune cell interaction exists and can be a factor in various disorders

Nerve-immune cell relationship in the central nervous system

In the past century, scientist have shown that not only do neurons and immune cells have their own distinct function, but these cells are able to interact with each other. Also, this interaction appears to be essential for daily functions throughout the body. With respect to the central nervous system which is composed of the brain and spinal cord, experiments reveal neurotransmitters and neuropeptides such as glutamate, norepinephrine, and vasoactive intestinal peptide can decrease immune responses within brain tissue (Neumann, 2001; Tian et al., 2009). Neurotrophic factors such as nerve growth factor and brain-derived neurotrophic factor can reduce immune function *in vitro* (Neumann, 2001). Also, pro-inflammatory cytokines released by immune cells can negatively modulate neurogenesis in the brain (Carpentier and Palmer, 2009).

In addition to the discovery of crosstalk between nerves and immune cells, an imbalance between these cells can be a factor for many neurodegenerative disorders. For example, inflammation is thought to contribute to the pathogenesis of multiple sclerosis, Parkinson's disease, and Alzheimer's disease (Tian et al., 2009; Cameron and Landreth, 2010; Chung et al., 2010; Wilson et al., 2010). Pro-inflammatory cytokines released by immune cells in addition to activated microglia (the brain's resident macrophage) can contribute to tissue damage in response to brain injury or stroke (Tian et al., 2009). These data suggest that crosstalk does exist between immune cells and the central nervous system and that this interaction must be balanced for proper immune and neural function.

Nerve-immune cell relationship in the peripheral nervous system

Autonomic

Interaction is also identified between the peripheral nervous system and immune cells. The peripheral nervous system is defined as the neurons and their axons outside of the central nervous system. The peripheral nervous system is divided into two groups: autonomic and somatic sensory. The purpose of the autonomic nervous system is to control involuntary functions such as breathing, digestion, and circulation. Research shows that the thymus, spleen, and bone marrow (all of which produce immune cells) are innervated by sympathetic nerve fibers, an autonomic nerve fiber subtype (Friedman and Irwin, 1997; Bellinger et al., 2008). Activation of sympathetic nerve fibers reduces immune cell proliferation *in vivo* (Pezzone et al., 1992; Friedman and Irwin, 1997).

Abnormalities in the relationship between the autonomic nervous system and immune cells have been linked to various disorders. For example, irritable bowel syndrome may be associated with inflammation-induced chronic excitation of enteric neurons, a subset of the autonomic nervous system (Lakhan and Kirchgessner, 2010). Our own laboratory has shown that immune cell infiltration after myocardial infarction can cause sympathetic hyperinnervation (Wernli et al., 2009). One topic that is gaining more attention, with respect to the autonomic nervous system, is the correlation between the extent to which a person is psychologically stressed and the intensity of an immune response (Irwin, 2008). Researchers hypothesize that changes in behavior can activate the autonomic nervous system which in turn decreases immunity (Irwin, 2008). Although most research is focused on neural influences on immune

cells, data show communication exists between the immune and autonomic nervous systems. Furthermore, crosstalk can be a modulator of immune function.

Somatic Sensory

Neurons of the somatosensory nervous system function to recognize thermal, mechanical, and painful stimuli and send information about said stimuli to the brain. Once processed, the brain in turn sends signals in an efferent direction to muscles to perform an action. With respect to nerve-immune cell interaction, bone marrow, spleen, and thymus are all innervated by sensory nerves that contain neuropeptides such as calcitonin gene-related peptide, vasoactive intestinal peptide, and substance P (Fink and Weihe, 1988; Weihe et al., 1989; Weihe et al., 1991). Studies reveal neuropeptides can reduce immune cell proliferation and function (Bulloch et al., 1995; Fox et al., 1997; Rochlitzer et al., 2011). Although the purpose is unclear, regulation of immune cell proliferation and function in normal tissue may be part of a feedback loop that prevents chronic inflammation.

Cutaneous disorders affect 1 in 3 individuals (Bickers et al., 2006). This can include psoriasis, dermatitis, acne, ulcers and infections. Annually, the United States spends billions of dollars to treat skin disorders (Bickers et al., 2006; Basra and Shahrukh, 2009). With respect to the epidermis of skin, crosstalk between nerves and immune cells is increasingly being recognized as a factor for cutaneous disorders. For example, inflammation associated with atopic dermatitis, psoriasis, and eczema is thought to be linked to hyperinnervation of sensory fibers (Chan et al., 1997; Jarvikallio et al., 2003; Tokime et al., 2008). Since immune cells can produce neurotrophins (Caroleo et al., 2001; Raap and Kapp, 2010; Yoshimura et al., 2010) and secrete cytokines that promote neurotrophin production through keratinocytes (Blasing et al., 2005), these data suggest immune cells may be inducing hyperinnervation. Taken together, the

relationship between the immune and nervous systems plays a role in nerve and immune cell status. Also, with respect to the pathogenesis of neurological and inflammatory disorders, this interaction is gaining more attention as a significant contributor to disease progression.

Components of the epidermis

Skin is the largest and one of the most important organs of the body. A major function of the skin is to protect our internal organs from external antigens and pathogens while providing information about various external stimuli. The epidermis, one of the components of skin, is the first line of defense against harmful pathogens and is often the site of abnormalities in nerve-immune cell interaction. In order to develop drugs that alleviate cutaneous complications associated with abnormal nerve-immune cell interactions, it is necessary to understand the cells that make up the epidermis and their function. Structurally, the epidermis has five layers: stratum corneum, stratum lucidum, stratum granulosum, stratum spinosum, and stratum basale. The epidermis is composed of cells such as keratinocytes, melanocytes, merkel cells, and migratory immune cells (Loser and Beissert, 2007; Boulais and Misery, 2008). Keratinocytes make up roughly 80% of the cells within the epidermis. Keratinocytes possess many functions within the skin such as providing growth factors for immune cells and nerve fibers (McKenzie and Sauder, 1990a; Boulais and Misery, 2008). In addition, they secrete many stimulatory factors which are necessary for immune responses and injury-induced inflammation (McKenzie and Sauder, 1990b).

Migratory immune cells such as macrophages, mast cells, neutrophils, T cells, and Langerhans Cells (LCs) can all be found in the epidermis (Loser and Beissert, 2007). Generally, these cells (except for LCs) are only found in the epidermis in response to foreign antigen invasion or inflammation associated with injury and wound healing (Cooper et al., 1993).

Macrophages, mast cells, neutrophils, and T-cells are phagocytic leukocytes. When an immune response occurs, these cells become activated and migrate to the site of the invasion to clear the region of foreign antigens. These immune cells also play an essential role in inflammation which is critical for proper wound healing (McKay and Leigh, 1991; Pradhan et al., 2009). Altogether, migratory immune cells play an important role in the overall function of the skin.

The epidermis is also innervated by intraepidermal nerve fibers (IENFs). IENFs are the sensory free nerve endings of axons that extend from the dorsal root ganglion and terminate in the epidermis (Jankowski and Koerber, 2010). These fibers are essential for recognizing external stimuli such as mechanical, thermal, and pain and sending information to the brain (Lumpkin and Caterina, 2007; Montano et al., 2010). IENFs are categorized into two subtypes: C and A-delta fibers. C fibers are unmyelinated slow-conducting fibers while A-delta fibers are finely myelinated and have an intermediate conduction rate (Jankowski and Koerber, 2010; Montano et al., 2010). Since they are activated by various stimuli, C and A-delta fibers are considered polymodal. C fibers can be further divided into two subtypes: peptidergic and non-peptidergic fibers. Peptidergic fibers secrete peptides such as calcitonin gene-related peptide, substance P, and vasoactive intestinal peptide (Karanth et al., 1991). Fibers that do not produce or secrete peptides (non-peptidergic) express isolectin-B4 (Gerke and Plenderleith, 2001). In terms of growth factors, peptidergic IENFs are highly responsive to nerve growth factor whereas non-peptidergic fibers are highly responsive to glial cell line-derived neurotrophic factor (Akkina et al., 2001; Montano et al., 2010). Overall, the epidermis is composed of different IENF subsets which allow the brain to distinguish various stimuli.

Intraepidermal nerve fiber and immune cells have a bi-directional crosstalk

Neuro-immune cell interaction has proven to be an essential part of inflammation associated with immune responses and injury in the skin. In 1919, scientists first showed inflammation induced by mustard oil application was reduced in denervated human skin (Breslauer, 1919; Jancso et al., 1967). Since then, numerous studies revealed that neuropeptides released by IENFs play a significant role with respect to inflammation. In addition, neurogenic inflammation is suggested to be critical component of wound healing (Pradhan et al., 2009). IENFs can also influence the intensity of an immune response (Steinhoff et al., 2003). A contact hypersensitivity model showed that after topical application of capsaicin the immune response was reduced (Beresford et al., 2004). Since capsaicin is known to deplete IENFs of neuropeptides, this suggests that neuropeptide are necessary for proper immune responses. When examining the effect of immune cells on IENFs, majority of research concentrates on macrophages, T cells, or mast cells. However, little is understood about the relationship between IENFs and LCs, the resident antigen presenting cells of the skin, and whether LCs influence IENFs.

Langerhans Cells

LCs were first discovered 143 years ago by Paul Langerhans (Langerhans, 1868). While making up only 2-3% of the epidermal cell population, these cells were first thought to be the intraepidermal nerve receptors (Jolles, 2002). Over a century later, researchers found that LCs were not neuronal receptors but possessed a phenotype similar to immune cells (Silberberg, 1973). Although LCs are present within stratified squamous epithelium of the oral cavity, vagina, esophagus, and cornea, (Rowden, 1967, 1981; Iijima et al., 2007) most information obtained about these cells is extrapolated from research pertaining to the epidermis.

LCs are thought to be involved in both the innate and adaptive immune response (de Jong and Geijtenbeek, 2010). In both cases, LCs can be activated by foreign antigens and/or co-stimulatory signals released by nearby keratinocytes (McKenzie and Sauder, 1990b). Upon activation, these cells endocytose foreign antigens and travel to nearby cutaneous lymph nodes where they present antigens to T cells. In response, T cells will travel to the site where the antigen was recognized and destroy the cells that express the foreign antigen (adaptive) or create memory T cells for any subsequent exposure to the same antigen (innate).

Under steady-state conditions, LCs are derived from haematopoietic precursor cells (Merad et al., 2008). However, research has also shown that under inflamed states circulating monocytes can modify their phenotype to that of LCs (Merad et al., 2008). LCs are distinguished from other immune cells by the presence of langerin and Birbeck granules (Merad et al., 2008). Langerin is a type II Ca^{2+} -dependent lectin (Valladeau et al., 1999; Valladeau et al., 2000; Romani et al., 2010). Langerin act as a “receptor” that binds to mannose expressing cells. Upon recognition, the ligand bound lectin is internalized for processing. Once processed, the cell presents small portions of the antigen with the use of the major histocompatibility complex II to T cells. Birbeck granules are a “tennis racket” like structure that are thought to be involved in antigen processing (Merad et al., 2008). These data suggests LCs play a critical role in the skin’s immune response system and potentially in injury-induced inflammation. A reduction in LCs may likely contribute to increased susceptibility to severe infections.

Crosstalk exists between Langerhans cells and intraepidermal nerve fibers

Most of the research available about LC-IENF interaction is focused on the effects of nerves on LCs. Many studies using different rat models have shown IENF degeneration

increases LC status in the epidermis (Hsieh et al., 1996; Lauria et al., 2005b). One study also revealed that LC status decreased once IENFs regenerate after nerve injury (Hsieh et al., 1996). Furthermore, LCs express neuropeptide receptors and upon binding decreases antigen presentation (Kodali et al., 2004; Ding et al., 2007). Given the role of LCs, these results suggest that a balance must be kept between LCs and IENFs for proper immune function.

Immune cells possess the ability to provide support for neurons (Vega et al., 2003; Lambiase et al., 2004; Kruse et al., 2007; Ralainirina et al., 2010). Although LC-like cells were able to cause PC12 cells to differentiate into neuronal-like cells *in vitro* (Torii et al., 1997), little is understood about whether LCs provide neurotrophic support *in vivo*. Within the epidermis, LCs are often in close proximity to and even appear to physically contact IENFs (Hosoi et al., 1993; Chateau and Misery, 2004). This physical arrangement could provide not only a way for IENFs to influence LCs but could also offer a way for LCs to promote IENF growth or alter IENF function. Therefore, the **objective** of the first study was to determine whether LCs influence IENFs in a LC depletion mouse model.

Nerve-immune cell interaction may be a factor in cutaneous complications associated with Diabetes Mellitus

Diabetes mellitus (DM) affects hundreds of million people worldwide of which over 20 million reside in the United States (Shaw et al., 2010). DM is characterized as a deficiency in insulin production, insulin action or both. If uncontrolled, DM can be a severely debilitating disease (Meijer et al., 2001). The American Diabetes Association states DM can lead to complications such as heart disease, stroke, high blood pressure, dental disease, kidney disease, blindness, complications with pregnancy, amputations, and neuropathy. With respect

to skin, common cutaneous complications associated with DM are small fiber neuropathy, cutaneous ulcers, and severely infected cutaneous wounds (Ferringer and Miller, 2002; Xie et al., 2010). Data reveal that contributing factors for the development of these complications can be ischemia, oxidative stress, an imbalance in the immune system, and reduction in neurotrophin expression within the epidermis (Zochodne, 2007). Although many factors contribute to the progression of diabetes-induced cutaneous complications, we are still unable to create therapeutic drugs that completely alleviate or prevent these issues. Since irregular nerve-immune cell interaction appears to be a factor for many cutaneous disorders, it is reasonable to hypothesize that abnormal nerve-immune cell interactions may also be a factor in diabetes-induced cutaneous complications. More specifically, the interaction between LCs and IENFs may prove to be a factor in the progression of cutaneous complications associated with diabetes. Therefore, the **objective** of the second study is to examine the relationship between LCs and IENFs in streptozotocin-induced diabetic rat.

Chapter 2: Langerhans Cells Regulate Cutaneous Innervation and Mechanical Sensitivity in the Mouse Footpad

Abstract

Intraepidermal nerve fibers are essential for detecting noxious and non-noxious stimuli. Since several disorders are associated with cutaneous axon degeneration, it is important to elucidate factors responsible for maintaining innervation. *In vitro* data suggest Langerhans cells, a major cutaneous antigen-presenting immune cell, produce neurotrophic factors, but it is unclear whether they contribute to skin innervation *in vivo*. We depleted Langerhans cells in Lang-DTR mice by diphtheria toxin administration and assessed cutaneous innervation, epidermal neurotrophin gene expression, and thermal and mechanical sensitivity in footpads. Following LC depletion, overall innervation immunoreactive for PGP9.5 was reduced by 28% in mice at 1 month of age. Axons immunoreactive for calcitonin gene-related peptide were also reduced by 43%, while immuno-negative axons were unaffected. In isolated epidermal sheets of LC-depleted mice, NGF and GDNF gene expression were markedly diminished, while BDNF was unchanged. Mice exhibited mechanical hypersensitivity, although thermal sensitivity was not altered. LC depletion at 10 months of age resulted in greater loss of PGP9.5-immunoreactive axons, due to selective depletion of axons immuno-negative for CGRP. Neurotrophin gene expression was unchanged at this age. These findings show LCs play a major role in regulating cutaneous neurotrophin gene expression and are critical in determining cutaneous innervation density and sensitivity in an age-dependent manner. Disturbances in LCs may therefore contribute to neurological dysfunction in peripheral neuropathies.

Introduction

Intraepidermal nerve fibers (IENFs) are sensory nerve endings of dorsal root ganglia that terminate in the epidermis (Lauria et al., 2009). Their function is to identify tactile, thermal, and noxious stimuli (Lumpkin and Caterina, 2007; Lauria et al., 2009), but they are important also for injury-induced inflammation (Steinhoff et al., 2003) and the skin's immune response to foreign antigens (Shepherd et al., 2005). Taken together, the multiple functions of IENFs make them a critical component of the skin. However, with the progression of certain disorders, such as diabetes (Lacomis, 2002; Hsieh, 2010), Guillain-Barré syndrome (Pan et al., 2003), and HIV/AIDS (Polydefkis et al., 2002), IENF degeneration can occur.

Mechanisms involved in maintaining IENFs remain unclear. Research shows that keratinocytes, a primary cell of the epidermis, produce neurotrophic factors (NTFs) (Di Marco et al., 1991; Yaar et al., 1991; Grewe et al., 2000; Marconi et al., 2003; Tokime et al., 2008; Montano et al., 2010; Truzzi et al., 2011). Overexpression of NTFs in keratinocytes increases IENFs suggesting that keratinocytes may be responsible for regulating IENFs.

However, keratinocytes are not the only cell in the epidermis that produce NTFs. Melanocytes, representing 5-10% of epidermal cells (Holbrook et al., 1988), also synthesize neurotrophic factors (Marconi et al., 2006). In addition, an *in vitro* study using a LC-like cell line suggests LCs produce NTFs (Torii et al., 1997). Langerhans cells (LCs) are considered the resident antigen-presenting cells of the epidermis (Ayala-Garcia et al., 2005). LCs capture foreign antigens and travel to regional lymph nodes where they present antigens to T cells, thus participating in innate and adaptive immune responses (Lore et al., 1998; Ayala-Garcia et al., 2005; de Jong and Geijtenbeek, 2010; Ginhoux and Merad, 2010). LCs are distinguishable from other epidermal cells by their ability to express langerin (Valladeau et al., 1999; Romani et al.,

2010) which is thought to be involved in ligand internalization and antigen presentation (Valladeau et al., 2000; Merad et al., 2008). There is reason to believe that LCs interact with IENFs. LCs make intimate contacts with IENFs (Hosoi et al., 1993; Gaudillere et al., 1996), and epidermal innervation appears to influence LC density (Hsieh et al., 1996; Stankovic et al., 1999; Siau et al., 2006; Jin et al., 2008). However, a role of LCs in epidermal innervation has not been explored. The objective of this study was to determine if LCs influence IENF density and their ability to respond to mechanical and thermal stimuli.

Materials and Methods

All animal protocols were approved by the University of Kansas Medical Center's Animals Care and Use Committee, and in accordance with the National Institute of Health guidelines for the care and use of laboratory animals.

Lang-DTR mice express the human diphtheria toxin receptor (DTR) fused to enhanced green fluorescent protein (eGFP) under the control of the langerin gene (Kissenpfennig et al., 2005). This allowed us to visualize and monitor LCs under various conditions and to temporarily deplete the skin of LCs by administering DT (Kissenpfennig et al., 2005; Bassett et al., 2011). We used Lang-DTR mice (a kind gift from Dr. Bernard Malissen) to examine the role of LCs in maintaining epidermal innervation in 1 and 10 month old mice.

DT Administration

LC depletion was accomplished by administering intraperitoneal injections of DT (1 μ g in 100 μ l, (Kissenpfennig et al., 2005)) (Sigma Aldrich, St. Louis, MO) or vehicle (water) on day 0 (first injection day), 4, 8, and 12. Mice were 1 or 10 \pm 1 month old when the experiment began. Twenty-three C57BL/6 (Jackson Laboratories, Sacramento, CA) and twenty-three Lang-DTR mice were used for this study.

Thermal and Mechanical Sensitivity

Thermal and mechanical sensitivity were measured one day prior to treatment and on post-injection days 1 and 13. Animals were placed upon a glass surface and allowed to acclimate for 30 minutes prior to thermal testing. Testing consists of a radiant heat source applied to the plantar surface of the hind paw, and time in seconds required for the animal to withdraw its paw (latency) is recorded. Thermal sensitivity was measured with a PAW Thermal Stimulator (4.3A; University of California San Diego, San Diego, CA). Each animal was tested 3 times on each hind paw with 5-10 minutes between each test.

After thermal testing, animals were placed upon a wire mesh platform and acclimated for 30 minutes before testing. An electronic von Frey apparatus (IITC Life Science, Woodland Hills, CA) was used to assess mechanical sensitivity by applying pressure to the hind paw with a monofilament. Pressure in grams required for the mouse to withdraw its paw was recorded. Each animal was tested 3 times with 5-10 minutes between tests.

Quantitative reverse transcription polymerase chain reaction

Quantitative reverse transcription polymerase chain reaction was performed to assess epidermal gene expression of nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), and glial cell line-derived neurotrophic factor (GDNF). On post-injection day 14, mice receiving DT or vehicle were anesthetized with isoflurane (Abbott Animal Health, Abbott Park, IL). The left hind paw footpad was excised and placed in 20mM EDTA for 1 hour at 37°C. Epidermal sheets were removed and rinsed in cold 0.1M phosphate buffered saline (PBS) for 5 minutes, frozen on dry ice, and stored at -80°C for mRNA extraction or immunostaining.

Total RNA was extracted in TRIzol (Invitrogen, Carlsbad, CA). Total RNA concentrations were measured using a NanoDrop ND-1000 spectrophotometer (ThermoScientific, Rockford, IL). To form complimentary DNA, 1µg of mRNA was used along with SuperScript II Reverse Transcriptase (Invitrogen, Carlsbad, CA) and MJ Mini Personal Thermal Cycler (BioRad, Hercules, CA). cDNA was amplified using iQ SYBR Green Supermix (BioRad, Hercules, CA) and an iCycler iQ Multicolor Real-Time PCR detection system (BioRad, Hercules, CA). Cycle number was recorded with the iCycler program (BioRad, Hercules, CA). Primer sets are: NGF, sense: TTTAAGAAACGGAGACTC and anti-sense: CTGTTGAAAGGGATTGTA; BDNF, sense: AGAGTGATGACCATCCTT and anti-sense: TGGACGTTTACTTCTTTCA; GDNF, sense: TTAAGTCCATACACTTA and anti-sense: CTACTTTGTCACTTGTTAG. For quantitation, glyceraldehyde 3-phosphate dehydrogenase was used as a housekeeping gene: sense: CTCTACCCACGGCAAGTTC and anti-sense: CTCAGCACCAGCATCACC (Integrated DNA Technologies, Coralville, IA).

Immunohistochemistry

Right hind paws were fixed in Zamboni's solution for 24 hours, rinsed daily in PBS for 14 days, immersed in 30% sucrose for 3-5 days, frozen in tissue freezing medium (Electron Microscopy Sciences, Hatfield, PA), and stored at -80°C. Footpads were serially sectioned at 20 µm in sagittal orientation. Sections were stored at -80°C prior to staining.

Sections were treated with 100mM glycine in PBS for 30 minutes followed by a 20 minute rinse in PBS and a 1 hour immersion in superbloc buffer (ThermoScientific, Rockford, IL). Tissue was stained overnight with primary antisera for protein gene product 9.5 (PGP9.5) (1:800, rabbit IgG, AbD Serotec, Raleigh, NC), calcitonin gene-related peptide (CGRP) (1:600, sheep IgG, Enzo Life Sciences, Farmingdale, NY), langerin (1:800, goat IgG Santa Cruz

Biotechnology, Santa Cruz, CA). After rinsing in PBS with 3% triton for 30 minutes, tissue was incubated with Cy3 conjugated donkey anti-goat (1:200), Cy3 conjugated donkey anti-rabbit (1:800), Alexa 488 conjugated donkey anti-rabbit(1:600), Cy3 conjugated donkey anti-sheep(1:400), or Cy2 conjugated donkey anti-goat IgG secondary antibodies (1:200) (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) for one hour. Sections were rinsed for 20 minutes in PBS and coverslipped with Fluoromount G. Three sections at 240µm intervals were analyzed per animal.

Epidermal Nerve Fiber Quantification

A method adopted by European Federation of Neurological Societies (Lauria et al., 2005a) was used to quantify IENFs. Individual IENFs crossing the dermal-epidermal junction were counted, excluding any secondary branching. Counts were divided by the length of the epidermis and expressed as IENF/mm. Three regions spaced equidistantly along the length of each section were selected, providing an index of innervation at the distal, middle, and proximal regions of the footpad. A 40X objective and 10X eyepiece on a Nikon Eclipse TE300 inverted microscope (Nikon Corp., Tokyo, Japan) was used to visualize IENFs. Each region spanned 0.42mm. To determine epidermal length for each region, images were taken and the basement membrane was measured. PGP9.5, a pan-neuronal marker (Wang et al., 1990), was used to identify both peptidergic and non-peptidergic IENFs. CGRP is a marker for peptidergic IENFs (Vaalasti et al., 1988) and was used to determine changes in CGRP-ir peptidergic IENFs. To determine non-peptidergic IENF density, CGRP-ir IENF density was subtracted from PGP9.5-ir IENF density. Non-peptidergic IENFs are referred to as CGRP-negative (-). Epidermal thickness was determined by measuring the height of the epidermis within the center of each image.

LC analysis in the footpad

To determine LC status in the epidermis, Z-stacks of confocal microscopic images of langerin immunoreactivity were captured at the distal, middle, and proximal regions each section with a 40X oil immersion objective on an Eclipse 90i microscope equipped with a Nikon C1si confocal system and a D-Eclipse camera (Melville, NY). Image renderings were created using maximum projection on volume renderings of Z-stacks using Nikon EZ-C1 FreeViewer (Melville, NY). Each image was calibrated and analyzed with Metamorph analysis program.

LC density was measured by dividing the number of LCs by the epidermal area (μm^2). Epidermal area was quantified by tracing around the epidermis, while excluding the stratum corneum layer. Research suggests LC size may be an indicator of functional maturity (Nishibu et al., 2006). For this reason, we measured LC size. Size was determined by dividing the langerin-ir area (μm^2) within the epidermis, measured by thresholding, by the number of LCs. To determine the percent of epidermal area occupied by LCs, langerin-ir area was divided by the epidermal area and multiplied by 100.

Statistical Analysis

Epidermal thickness, LC density, size, the percent epidermal area occupied by LCs, IENF density, and gene expression was analyzed statistically by t-test or two way analysis of variance and Student-Newman-Keuls post-hoc analysis. Behavioral data was analyzed using two way repeated measures analysis of variance and Student-Newman-Keuls post-hoc analysis. Differences were considered significant if p value was ≤ 0.05 . Data are expressed as Mean \pm Standard Error Mean (SEM).

Results

Diphtheria toxin does not affect LCs in C57BL/6 mice

We confirmed that DT, a foreign antigen and potential toxin, does not affect skin and innervation in normal mice. DT did not affect epidermal thickness (Table 1), nor did it alter LC density, size, or the % of epidermal area occupied within 1 or 10 month old mice (Figure 1). DT had no effect on PGP9.5-ir (Figure 2a-b and i), CGRP-ir (Figure 2c-d and j), and non-peptidergic (Figure 2k) innervation of 1 month old C57BL/6 mice. However, in 10 month old mice, DT reduced PGP9.5-ir IENFs by 22% ($p=0.007$, Figure 2e-f and i), without a detectable effect on peptidergic or non-peptidergic fibers (Figure 2g-h and j-k).

LC depletion reduces IENF density

DT administration to Lang-DTR mice depleted LCs in the footpad, as previously described for ear (Kissenpfennig et al., 2005). Langerin-ir LCs were present in the epidermis of vehicle-treated Lang-DTR mice (Figure 3a-c) but absent after DT treatment (Figure 3d-f). LC depletion did not affect epidermal thickness in 1 and 10 month old Lang-DTR mice (Table 1).

To determine if LC depletion alters cutaneous innervation, IENF density was quantified. LC depletion in 1 month old Lang-DTR mice, caused a 28% decrease in PGP9.5-ir IENF density ($p=0.036$, Figure 4a, b, and e). LC depletion reduced CGRP-ir IENFs by 43% ($p=0.045$, Figure 4c-e), while non-peptidergic IENF density was unchanged (Figure 4e).

Langerhans cells and intraepidermal nerve fibers are reduced in older Lang-DTR mice

Aging is associated with reductions in LC content (Bhushan et al., 2002; Cumberbatch et al., 2002) which may be a confounding factor when determining their role in maintaining IENFs. Accordingly, we assessed whether LCs were altered in older vehicle-treated mice. LC density

was reduced by 31% in older mice (677 ± 65 vs. 465 ± 21 # of LCs/mm² in 1 and 10 month old mice, respectively) ($p=0.022$, Figure 5a and b). There was no significant change in LC size (185 ± 18 vs. 182 ± 8 μm^2 /# of LCs, Figure 5) or the % of epidermal area occupied by LCs (10 ± 1 vs. $8 \pm 1\%$, Figure 5). Also, age did not affect epidermal thickness in vehicle-treated Lang-DTR mice (Table 1).

IENFs are also reported to decrease with age (Goransson et al., 2004; Lauria et al., 2010). PGP9.5-ir IENF density was reduced by 28% in older vehicle-treated mice ($p=0.021$, Figure 4a and 6a). CGRP-ir fibers tended to be lower in older vehicle-treated mice, although this did not achieve statistical significance ($p=0.058$, Figure 4c and 6c). Non-peptidergic IENF density was unchanged.

IENF loss after LC depletion is greater in older mice

We examined if IENF loss after LC depletion was altered in older mice. There appeared to be fewer PGP9.5-ir IENFs in LC-depleted mice at 10 months of age (Figure 6b). Quantitative analysis revealed a 52% decrease in PGP9.5-ir IENF density after DT administration ($p<0.001$, Figure 6e) which was significantly greater than the reduction seen in 10 month old DT-treated C57BL/6 mice ($p<0.001$). When compared to 1 month old mice, PGP9.5-ir IENFs were reduced by 52% ($p=0.001$, Figure 4e and 6e). CGRP-ir IENFs were not altered by LC depletion in 10 month old mice (Figure 4c-e), and, there was no difference in CGRP-ir IENF density between 1 and 10 month old LC-depleted mice (Figure 4d and 6d). However, LC depletion caused a 98% decrease in non-peptidergic IENFs at 10 months ($p=0.007$, Figure 6e). When compared to 1 month old LC-depleted mice, 10 month old mice lacking LCs had a 98% reduction in non-peptidergic IENFs ($p=0.014$, Figure 4e and 6e).

Changes in epidermal neurotrophic factor gene expression after LC depletion

To determine if IENF loss after LC depletion is accompanied by reductions in NTF gene expression, we analyzed epidermal sheets of 1 and 10 month old mice using quantitative reverse transcription polymerase chain reaction (Figure 7). The epidermis is known to produce BDNF, NGF, and GDNF which influence large sensory (LeMaster et al., 1999), small peptidergic (Donnerer et al., 1996; Rice et al., 1998), and small non-peptidergic fibers (Akkina et al., 2001; Adly et al., 2006), respectively. No significant change in BDNF expression was observed with LC depletion in 1 month old Lang-DTR mice (Figure 7a). However, NGF (Figure 7b) and GDNF (Figure 7c) gene expression were reduced ($p=0.012$ and $p=0.015$, respectively). The fold reduction implies that LC depletion results in roughly a 75% loss of NGF and 90% GDNF gene expression at 1 month. However, NTF gene expression was not altered in 10 month old LC-depleted Lang-DTR mice (Figure 7a-c), and DT did not affect NTF gene expression in C57BL/6 mice (data not shown).

Allodynia occurs after LC depletion

Abnormal sensitivity occurs in many cutaneous disorders associated with nerve loss (Lin et al., 2001; Johnson et al., 2008; Ward et al., 2011). Since LC depletion resulted in IENF loss, we assessed whether mechanical (Figure 8) and thermal sensitivity was altered. We observed no differences in mechanical sensitivity at baseline and post-injection day 1 and 13 within vehicle- or DT-treated C57BL/6 mice (Figure 8a). Also, DT did not alter mechanical sensitivity in C57BL/6 mice. Analysis showed mechanical sensitivity was not altered in vehicle-treated Lang-DTR mice. However, LC-depleted mice exhibited increased mechanical sensitivity at day 13 when compared to pre-injection controls and all other groups at post-injection day 13 ($p<0.001$). Vehicle- and DT-treated C57BL/6 and Lang-DTR mice did not exhibit any changes in thermal sensitivity (data not shown).

Discussion

Many cell types influence epidermal innervation. This includes keratinocytes which produce a variety of neurotrophic factors. If overexpressed, these factors can result in hyperinnervation. In addition, immune cells, which are critical for immune responses and promoting inflammation, produce neurotrophic factors and cytokines that can modulate innervation (Caroleo et al., 2001; Blasing et al., 2005; Raap and Kapp, 2010; Yoshimura et al., 2010). Until now, LCs are generally seen as antigen-presenting cells involved in innate and adaptive immune responses. It is with the use of Lang-DTR mice, we show that LC depletion induced by DT administration causes IENF loss resulting in a new role for LCs as a regulator of epidermal innervation.

IENFs, which can be divided into C and A- δ fibers, detect thermal, mechanical, and noxious stimuli. C fibers are further categorized as peptidergic and non-peptidergic. Since these fibers detect multiple stimuli, they are considered polymodal (Perl, 1996). A reduction in a specific fiber subtype may explain changes observed in cutaneous thermal and mechanical sensitivity. CGRP-containing nerves are peptidergic and generally responsive to thermal stimuli (Jankowski and Koerber, 2010). Therefore, we expected to find a loss of CGRP-ir nerves to be accompanied by abnormal thermal sensitivity. However, CGRP-ir IENF loss was associated with increased mechanical sensitivity in 1 month old LC-depleted mice. Mice could be hypersensitive to mechanical stimuli because the remaining IENFs (non-peptidergic) become hypersensitive due to an imbalance between peptidergic and non-peptidergic IENFs. Keratinocytes can influence cutaneous sensitivity (Schmelz, 2011) and LCs communicate with keratinocytes through cytokine signaling (McKenzie and Sauder, 1990b; Matsue et al., 1992; Parkinson et al., 1993). Therefore, a lack of interaction between LCs and keratinocytes may cause keratinocytes to alter sensitivity. Nevertheless, our data show LCs provide trophic support

for CGRP-producing nerves that are required for mechanical sensitivity. Further, these data support previous studies that found allodynia to be associated with cutaneous neurodegeneration (Sommer and Schafers, 1998; Renn et al., 2011a; Renn et al., 2011b; Ward et al., 2011).

We determined that LCs are necessary for maintaining epidermal innervation, but by what mechanism do LCs provide support? Neurodegeneration is often associated with a reduction in neurotrophic factors (Anand et al., 1991; Pittenger and Vinik, 2003). Since neurotrophic factors such as NGF and GDNF support CGRP-ir and non-peptidergic nerves (Lindsay, 1996; Montano et al., 2010), we examined if IENF loss was associated with a reduction in NGF and GDNF. BDNF influences large fiber mechanoreceptors. A reduction in BDNF may result in altered mechanical sensitivity. Therefore, BDNF was also measured. LC depletion in 1 month old mice was accompanied by a 75% and 95% reduction in NGF and GDNF, respectively. This implies that LCs regulate a substantial amount of NGF and GDNF expression in the epidermis. BDNF was unaltered suggesting that large fiber mechanoreceptors may not be altered. Due to the fact that epidermal cells, mostly keratinocytes, produce NTFs we needed to confirm that NTF reduction after LC depletion was not caused by a reduction in epidermal content. LC depletion did not alter epidermal content implying that LCs provide trophic support for CGRP- containing nerve fibers by modulating NGF and GDNF gene expression in 1 month old mice.

There are two potential mechanisms for how NGF and GDNF levels changed. First, NGF mRNA has been identified in a LC-like cell line (Torii et al., 1997). If LCs produce NTFs *in vivo*, a reduction in LCs would cause a decrease NTF expression. Second, the epidermis is primarily composed of keratinocytes, which secrete NTFs, and LCs and keratinocytes communicate with each other. It is possible that a lack of interaction between LCs and nearby keratinocytes results

in decreased NTF expression which leads to IENF reduction. Clearly, future studies are required to determine how LCs alter NTF expression in 1 month old mice.

LCs and IENFs are reduced with aging suggesting the relationship between LCs and IENFs may be different with age. Aging reduced LCs and PGP9.5-ir IENFs. Small but non-significant reductions in CGRP-ir and non-peptidergic IENFs were observed with age indicating PGP9.5-ir IENFs reduction is due to both subtypes being affected. Earlier, we established that LCs are essential for maintaining IENFs. It is likely that the reduction in IENFs in older mice was due to a reduction in LCs. Since age did influence LCs and IENFs, we investigated whether IENF loss after LC depletion was altered in older mice. However, first it was necessary to determine if DT influenced IENFs in older mice. PGP9.5-ir IENFs were reduced in 10 month old C57BL/6 mice. To assess if this reduction is due to DT affecting LCs, we examined LCs in 10 month old C57BL/6 mice. DT did not alter LCs in older C57BL/6 mice suggesting the reduction in IENFs is not a response to changes in LCs but to DT administration. Although it remains unclear how DT reduced IENFs and why it only occurred in older mice, a potential mechanism could be through CD9, which is expressed in the peripheral nervous system of mice (Schmidt et al., 1996) and known to promote DT/DTR binding (Nakamura et al., 1996). These data imply that age influences LCs and IENFs whereas DT only alters IENFs. Moreover, analysis of epidermal thickness reveal changes in LCs and IENFs with age and DT is not due to reduced epidermal content.

We revealed age and DT are factors when assessing IENFs and LCs status. While taking these factors into consideration, IENF density was analyzed in older LC-depleted mice. When compared to 1 month old LC-depleted mice, PGP9.5-ir IENFs were further reduced. This was also significantly lower than the reduction found in C57BL/6 mice suggesting LC depletion caused IENF degeneration. There are multiple explanations for why a greater effect was

observed in older mice. First, DT reduces innervation in older mice. As a result, a greater decrease may be a combined effect of LC depletion and DT. Second, LCs were reduced in older vehicle-treated mice. Therefore, a greater decrease may be due to the fact that there was less trophic support by LCs to begin with and when removed this caused an even greater reduction. In any case, differences in innervation are not due to changes in epidermal content which was reflected in epidermal thickness. Quantitative analysis of IENF subtypes revealed peptidergic IENFs were not altered while the epidermis was almost completely absent of non-peptidergic IENFs. This suggests LCs provide trophic support for non-peptidergic IENFs in older mice. Due to a limitation of resources, we were unable to assess sensitivity in older mice. Since non-peptidergic IENF loss has been associated with increased sensitivity to noxious stimuli (Golden et al., 2010), it is possible that LC depleted mice may be more sensitive to noxious stimuli.

With age the IENF subtype supported by LCs switched from CGRP-producing IENFs to non-peptidergic IENFs suggesting the mechanism used to support IENFs may have changed. Therefore, NTF gene expression was examined. Despite the dramatic decrease in IENF density in older mice, BDNF, NGF, and GDNF levels were not altered by LC depletion. It is possible that NTF levels did not change because LC's influence on NTF expression is stronger in younger mice. Also, LCs could provide trophic support through other neurotrophic factors or cytokine signaling such as interleukin-1 β (IL-1 β). Since LCs produce IL-1 β (Matsue et al., 1992) and IL-1 β increases neurite outgrowth in culture (Edoff and Jerregard, 2002), LCs may be directly affecting IENFs through IL-1 β . Altogether, data from older mice suggest LCs use a different pathway to provide trophic support for non-peptidergic IENFs which requires further investigation.

Collectively, we reveal that LCs play a pivotal role in maintaining epidermal innervation. Yet, age-dependent changes in NTF expression indicate the process by which LCs provide support involves multiple pathways. Despite this conundrum, our study provides evidence that the relationship between LCs and epidermal nerve fibers is a two-way street and that future studies are needed to determine how LCs help maintain IENFs in older mice. This data adds another level to the already complex nerve-immune cell relationship within the skin. With respect to peripheral neuropathy, it remains unclear what steps occur prior to neuropathy. However, data from this study suggest that LC reduction results in IENF loss and hypersensitivity which may mean peripheral neuropathy and cutaneous hypersensitivity associated with diseases may be induced or exacerbated by a loss of LCs. This data can also be useful in understanding nerve regeneration during wound healing. Since LCs are necessary for maintaining IENFs, LCs may need to be present to obtain full nerve regeneration. Altogether, our data provides evidence of a new role for LCs.

Tables

Table 1 Epidermal Thickness in C57BL/6 and Lang-DTR Mouse Footpad

	C57BL/6	Lang-DTR
<u>1 Month</u>		
Vehicle	58.14 ± 4.92	59.91 ± 6.82
DT	50.82 ± 4.51	51.99 ± 2.19
<u>10 Month</u>		
Vehicle	64.11 ± 3.14	67.89 ± 2.45
DT	66.28 ± 4.39	66.55 ± 3.33

Table 1 Epidermal thickness in C57BL/6 and Lang-DTR mice

Epidermal thickness was measured in footpad sections from C57BL/6 and Lang-DTR mice treated with vehicle or DT at 1 and 10 months of age. Data is expressed as Mean \pm SEM.

Figures

Figure 1

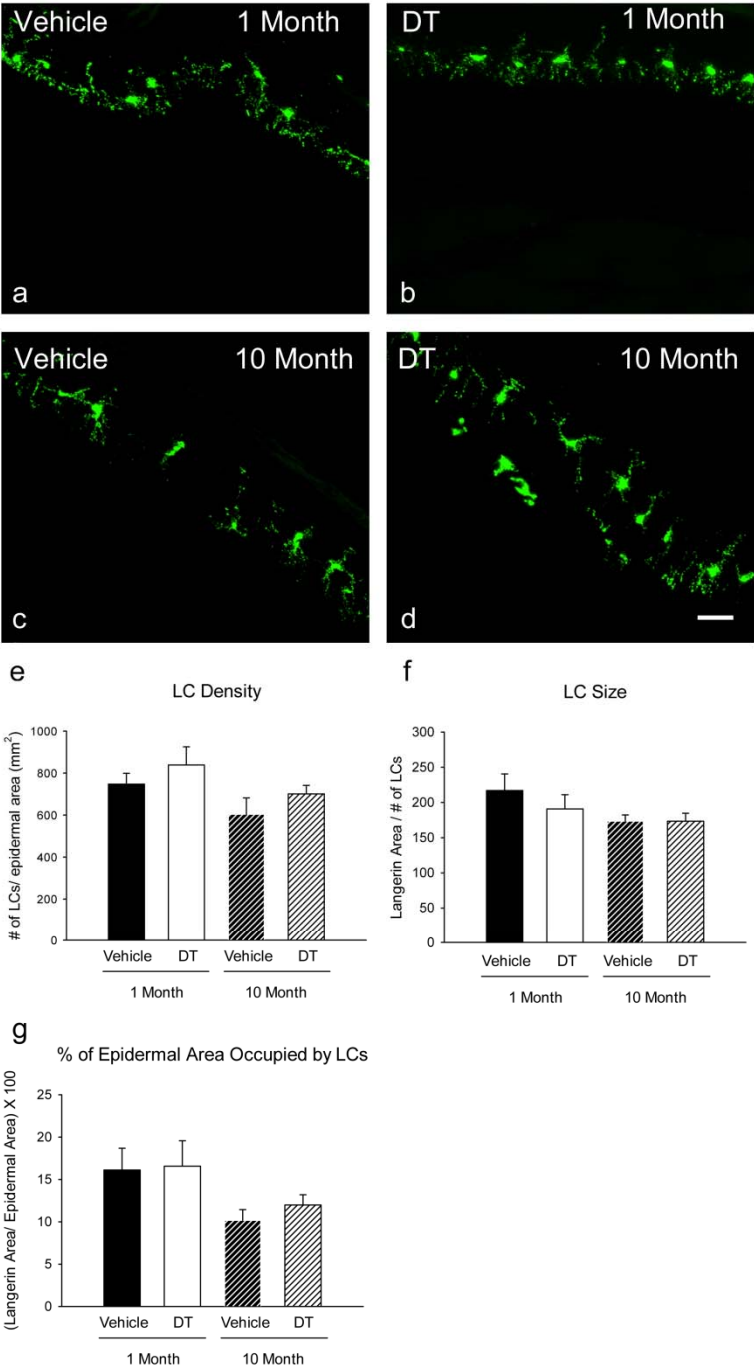


Figure 1 LC status in C57BL/6 mice

Footpad sections from C57BL/6 mice at 1(a and b) and 10 (c and d) months of age were stained for langerin (a-d) after treatment with vehicle (a and c) or DT (b and d). Images were taken with a 60x objective. Bar graphs represent LC density (e), LC Size (f), and the % of epidermal area occupied by LCs (g). Scale bar = 20 μ m. Data is expressed as Mean \pm SEM.

Figure 2

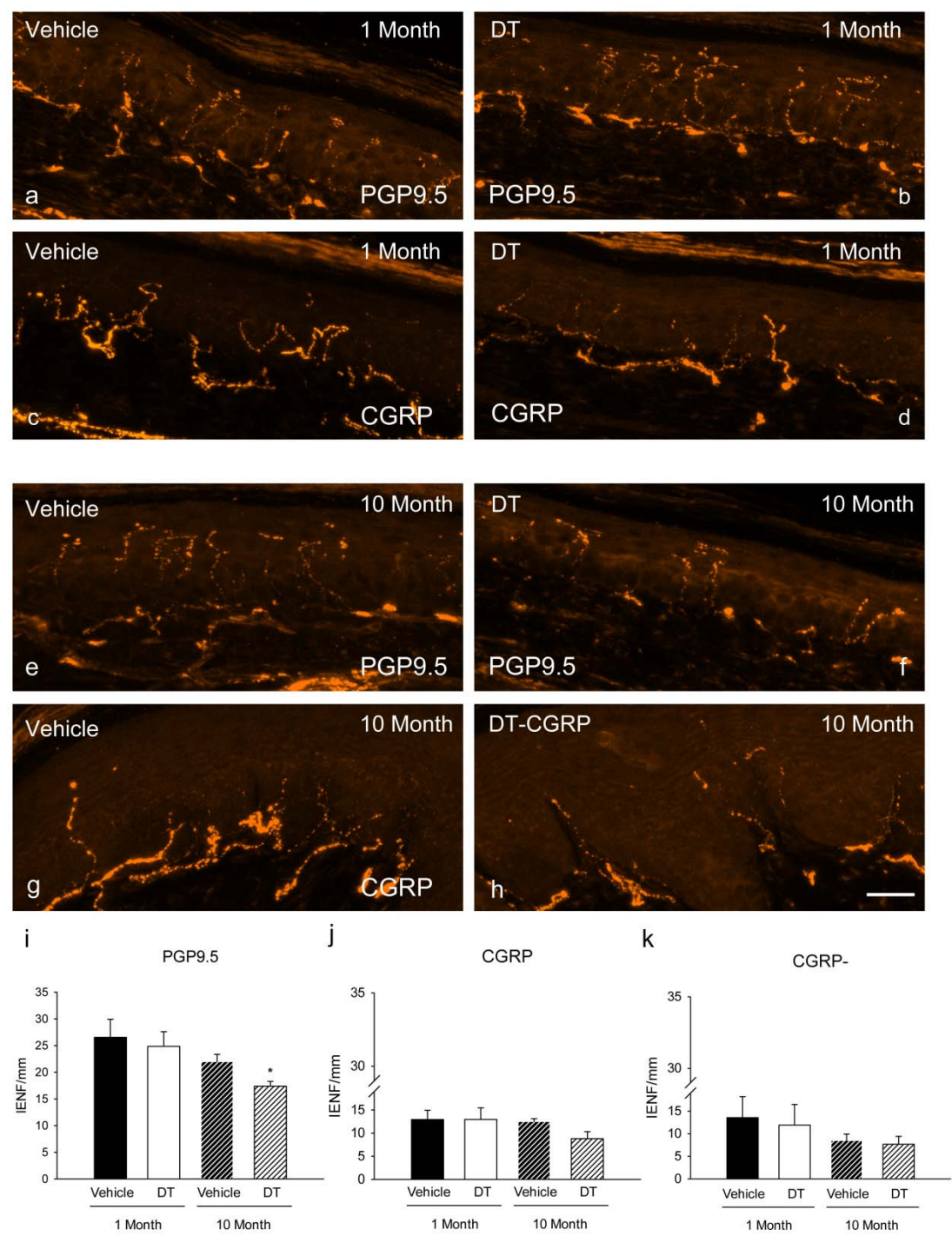


Figure 2 PGP9.5-ir, peptidergic, and non-peptidergic IENFs in C57BL/6 mice

Footpad sections from 1 and 10 month old (a-d and e-h, respectively) vehicle- (a, c, e, and g) or DT-treated (b, d, f, and h) C57BL/6 mice were stained for PGP9.5 (a, b, e, and f) and CGRP (c, d, g, and h) to reveal total IENFs and IENFs within the peptidergic subtype, respectively. Images were taken with a 60x objective. Scale bar represents 20 μ m. Bar graphs represent PGP9.5-ir (i), CGRP-ir (j), and CGRP(-)-ir (non-peptidergic, k) IENF density. *p=0.007 when PGP9.5-ir IENF density is compared between aged vehicle- and DT-treated mice. Data is expressed as Mean \pm SEM.

Figure 3

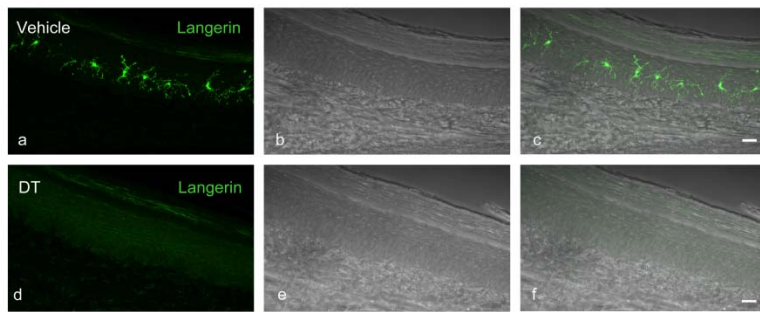


Figure 3 LC depletion in Lang-DTR mice

Images of footpad sections from vehicle- (a-c) and DT-treated (d-f) Lang-DTR mice immunostained for langerin were taken with a 40x objective. Transmitted light images reveal the morphology of the epidermis in vehicle- (b) and DT-treated (e) mice. Scale bars in c and e represent 20µm. Data are represented as Mean ± SEM.

Figure 4

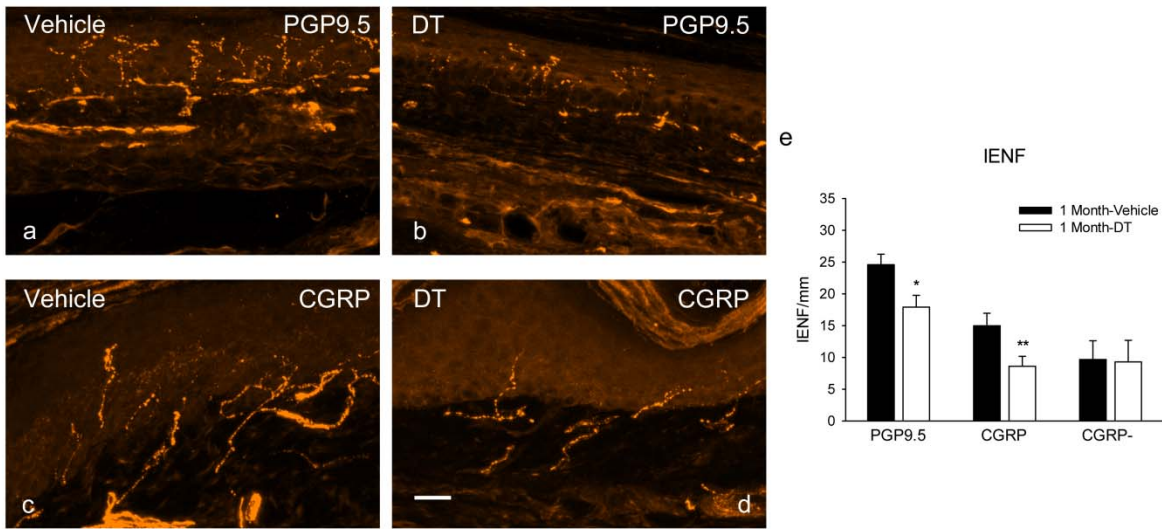


Figure 4 Epidermal innervation in 1 month old Lang-DTR mice

Images of PGP9.5-ir (a and b) and CGRP-ir (c and d) IENFs in the footpad of 1 month old vehicle- (a and c) and DT-treated (b and d) Lang-DTR mice were taken with a 60x objective. Scale bar represents 20 μ m. Bar graph represents PGP9.5-ir, CGRP-ir, and CGRP(-)-ir IENF density in 1 month old Lang-DTR mice. * $p=0.036$ when PGP9.5-ir IENF density for vehicle- and DT-treated mice are compared. ** $p=0.045$ when CGRP-ir IENF densities are compared between vehicle- and DT-treated mice. Data is represented as Mean \pm SEM.

Figure 5

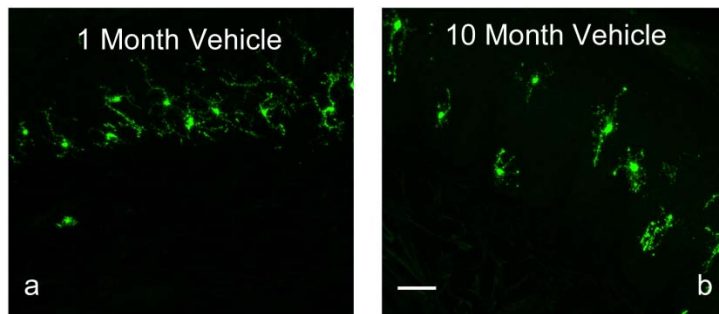


Figure 5 LCs in 1 and 10 month old Lang-DTR mice

Images reveal langerin immunoreactivity in footpad sections of 1 and 10 month old Lang-DTR mice (a and b, respectively). Scale bar represents 20 μ m.

Figure 6

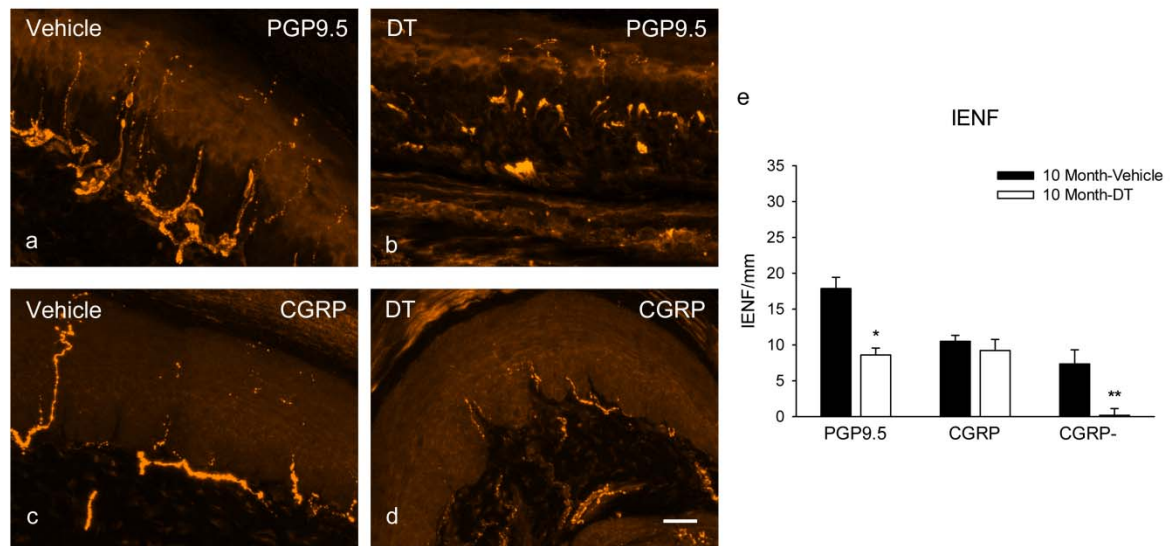


Figure 6 Epidermal innervation in older Lang-DTR mice

(a) and (b) reflects PGP9.5-ir IENFs in vehicle- and DT-treated 10 month old Lang-DTR mice, respectively. (c) and (d) show CGRP immunoreactivity in footpad sections of vehicle- and DT-treated 10 month old Lang-DTR mice, respectively. All images were taken with a 60x objective. Scale bar represent 20 μ m. Bar graph represents PGP9.5-ir, CGRP-ir, and CGRP(-)-ir IENF density in 10 month old Lang-DTR mice. * $p < 0.001$ when older vehicle- and DT-treated mice are compared. ** $p = 0.007$ when older vehicle- and DT-treated mice are compared. Data is represented as Mean \pm SEM.

Figure 7

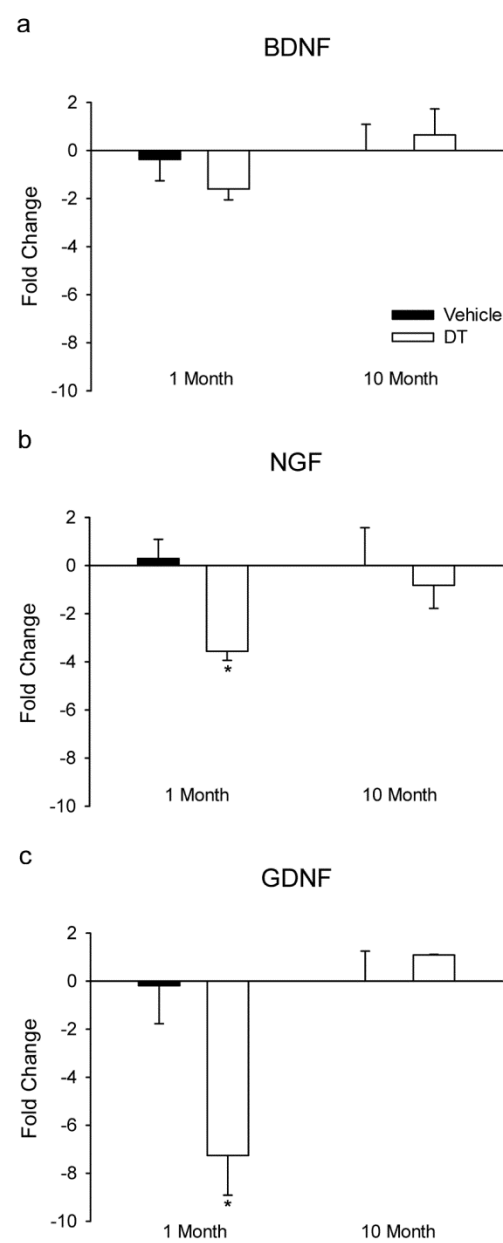


Figure 7 Neurotrophic factor expression in epidermal sheets of Lang-DTR mice

Bar graphs represent fold changes in BDNF (a), NGF (b), and GDNF (c) relative to controls.

Gene expression was made in both 1 and 10 month old mice. * $p=0.012$ when vehicle- and DT-treated mice are compared at 1 month of age. ** $p=0.015$ when 1 month old vehicle-and DT-treated mice are compared. Data is expressed as Mean \pm SEM.

Figure 8

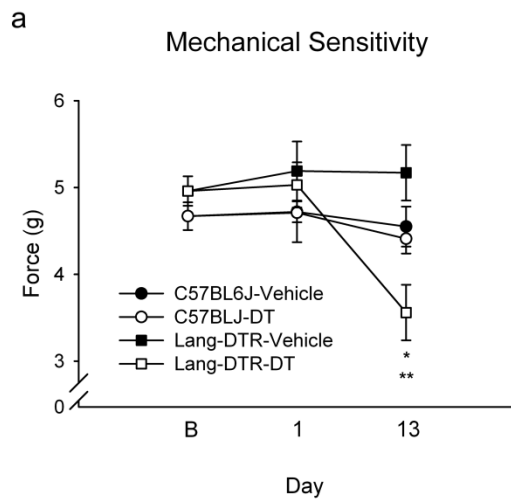


Figure 8 Mechanical sensitivity in 1 month old Lang-DTR mice

Line graph (a) represents mechanical sensitivity. Measurements were taken one day prior to DT treatment (B) and one (day1) and thirteen (day13) days after the first injection. Filled and open circles represent vehicle- and DT-treated C57BL/6 mice, respectively. Filled and open squares represent vehicle- and DT-treated Lang-DTR mice. * $p < 0.001$ when DT-treated Lang-DTR mice on day13 are compared to their baseline. ** $p < 0.001$ when DT-treated Lang-DTR mice are compared to vehicle-treated Lang-DTR on day13. Data is expressed as Mean \pm SEM.

Chapter 3: Nerve-Langerhans Cell Interactions in Diabetes and Aging

Abstract

Cutaneous infections are a leading cause of hospitalization in diabetic patients. Langerhans cells (LCs) are antigen-presenting cutaneous dendritic cells that protect against infections, but the effect of diabetes and aging on these cells is unclear. We examined LCs in footpads of rats with streptozotocin-induced diabetes at 3 months of age following 4 weeks of diabetes, and at 6 months following 16 weeks of diabetes. Immunostaining of LCs using the selective marker protein langerin showed LC surveillance increased between 3 and 6 months of age as a result of increased LC numbers and size in control rats. In diabetic rats, LC numbers increased with age but, unlike 6 month old controls, cell size did not, suggesting that diabetes impairs the increase in cell size that is a hallmark of LC maturation. Immunostaining also revealed that diabetes reduced LC numbers after 4 weeks and numbers and sizes following 16 weeks. We examined the relation between LC and innervation and found that, while axon density decreased with aging, it was not affected by diabetes. However, LCs expressing the neuronal marker PGP9.5 represented a source of error in axonal counts. These findings support the hypothesis that diabetes substantially impacts LC proliferation and maturation independent of effects on cutaneous innervation. Accordingly, the interactions of diabetes and aging on LCs may be important factors in predisposing diabetic patients to cutaneous ulcers and infections.

Introduction

Infection of cutaneous ulcers is a major cause for hospitalization and amputation among the diabetic population (Reiber et al., 1998; Cruciani et al., 2009; Xie et al., 2010). Although the incidence of cutaneous infections is not greater (Oumeish, 2008), healing time is delayed and morbidity more severe (Ferringer and Miller, 2002; Xie et al., 2010). The reasons for the greater frequency of ulcer pathologies in diabetes is unclear, but accompanying neuropathies and angiopathies may well contribute to delayed healing (Meijer et al., 2001; Wohlrab et al., 2007; Ghanassia et al., 2008; Lauterbach et al., 2010). In addition, the skin contains an intrinsic immune system that helps protect against infection. Antigen presenting cells (APCs) which include macrophages, B cells, and dendritic cells, and are critical for preventing foreign antigen infiltration. Impairments in the cutaneous immune system could possibly contribute to the poor outcome in diabetics developing cutaneous ulcers.

The main APC in the epidermis is the Langerhans cell (LC). LCs are stellate dendritic cells derived from hematopoietic precursors (Merad et al., 2002), and are also found within stratified squamous epithelium in other body regions (Pieri et al., 2001; Merad et al., 2008) including the oral cavity (Rowden, 1981), esophagus (Rowden, 1967), and vagina (Iijima et al., 2007). LCs express antigen-presenting proteins such as major histocompatibility complex (MHC)- I and II, human leukocyte antigen-DR (HLA-DR), and CD1a (Harrist et al., 1983; Ayala-Garcia et al., 2005; Mutyambizi et al., 2009; Romani et al., 2010), and their primary function is believed to be to present atypical self or foreign antigens to T cells (Merad et al., 2008; Zaba et al., 2009). LCs are also characterized by the presence of the type II Ca^{2+} -dependent lectin, langerin (Valladeau et al., 1999; Valladeau et al., 2000; Romani et al., 2010). Langerin has a carbohydrate recognition domain that specifically binds to and is involved in uptake of mannose-expressing antigens (Valladeau et al., 2000; Chatwell et al., 2008). The primary function of

langerin is thought to be ligand internalization associated with Birbeck granule formation, a feature exclusive to epidermal LCs (Valladeau et al., 1999; Valladeau et al., 2000; Kissenpfennig et al., 2005). Consequently, these cells are important in cutaneous immune responses (Mutiyambizi et al., 2009; Romani et al., 2010).

Diabetes could influence LCs through direct effects on cell maturation or longevity, or indirectly through microvascular changes (Jeffcoate and Harding, 2003; Wohlrab et al., 2007) or altered keratinocyte growth factor production. Further, intraepidermal nerve fibers (IENFs) appear to regulate LC composition, as peripheral degeneration increases LC numbers (Hsieh et al., 1996; Stankovic et al., 1999; Lindenlaub and Sommer, 2002; Lauria et al., 2005b; Siau et al., 2006; Jin et al., 2008). Since neuropathic changes are common in diabetes (Lauria et al., 2005b; Lauria and Lombardi, 2007; Beiswenger et al., 2008; Sommer, 2008; Nebuchennykh et al., 2009), it is important to assess whether changes in IENFs may be a factor mediating diabetes' effect on LCs.

Thus far, two studies have quantified the effect of diabetes on LCs, yielding conflicting results (Ziegler and Standl, 1988; Lauria et al., 2005b). These studies examined skin from different locations, species, and at different times after the onset of diabetes using either MHC-II or protein gene product 9.5 (PGP9.5) as a LC marker. PGP9.5 is expressed by LCs (Hsieh et al., 1996; Stankovic et al., 1999; Lin et al., 2001; Lauria et al., 2005b; Beiswenger et al., 2008; Jin et al., 2008), but is also present within IENFs and commonly used to quantify innervation (Jackson and Thompson, 1981; Lauria et al., 2005b; Lauria and Lombardi, 2007; Beiswenger et al., 2008; Sommer, 2008; Lauria et al., 2009; Nebuchennykh et al., 2009). Since LCs form intimate associations with IENFs, questions have emerged regarding whether PGP9.5-immunoreactive (ir) LCs may be a confounding factor in quantifying IENFs.

The purpose of this study was to systematically examine the effect of different durations of streptozotocin-induced diabetes and, concurrently, the effects of age on epidermal LCs and innervation using dual staining for PGP9.5 and langerin. Our objective was to identify cellular mechanisms that could contribute to the more profound incidence and severity of cutaneous infections and cutaneous ulcerations in diabetic patients.

Material and Methods

All animal protocols were approved by the University of Kansas Medical Center's Animals Care and Use Committee, and were in accordance with the National Institute of Health guidelines for the care and use of laboratory animals.

Sixty-day old female Sprague Dawley rats (Harlan Laboratories, Inc., Indianapolis, IN) were anesthetized (60mg/kg ketamine HCL, Ketaject, 0.4 mg/kg atropine sulfate, and 8 mg/kg xylazine Xyla-Ject, intraperitoneal (i.p.) injection) and bilaterally ovariectomized under aseptic conditions (Blacklock and Smith, 2004). Estrogen is known to alter LC numbers (Koyama et al., 1987; Hernandez-Segura et al., 2005; Kovats and Carreras, 2008), and reproductive hormone levels vary with age and reproductive status in both males and females. Therefore, using females with constant and very low levels of estrogen after ovariectomy (Strom et al., 2008) eliminates a significant confounding variable.

Diabetes Induction

One week following ovariectomy, blood samples were taken from the tail vein and blood glucose was measured with a TrueTrack Smart System glucometer (Ft Lauderdale, FL). Animals then received a single i.p. injection of streptozotocin (STZ, 60mg/kg, in 10mM citrate

buffer with 0.9%NaCl, pH4.5, (n=17) or vehicle buffer (n=11). Three days following STZ administration, blood glucose levels were again assessed, and animals with levels ≥ 270 mg/dl were considered diabetic and included in the experiment. Animals were maintained for 4 (n=6) or 16 weeks (n=11) at which time, blood glucose and weight were measured.

Immunostaining

Animals were anesthetized with Beuthanasia (195mg/kg Sodium Pentobarbital, 25mg/kg Phenytoin Sodium, i.p.;Schering-Plough Animal Health Corp., Union, NJ) and footpads were harvested. Tissue was immediately immersed in Zamboni's fixative for 24-hours. Tissue was rinsed daily in 0.1M phosphate buffered saline (PBS) for 10-14 days. After rinsing, footpads were immersed in 30% sucrose solution. Tissue was frozen in tissue freezing medium (Electron Microscopy Sciences, Hatfield, PA) and stored at -80°C. Footpads were serially sectioned at 18 μ m thickness perpendicular to the surface and along the length of the footpad. Three sections spaced 342 μ m were analyzed per animal.

Sections were stained with goat anti-langerin antiserum (1:100, Santa Cruz Biotechnology, Santa Cruz, CA,) to identify epidermal LCs. Immunofluorescence with the langerin antibody in tissue from mice expressing enhanced green fluorescent protein under the control of the langerin gene (kindly provided by Dr. Bernard Malissen) confirmed that our langerin antibody was specific for Langerhans cells. Also, sections were stained with a rabbit anti-human PGP9.5 antibody (1:600, AbD Serotec, Oxford, UK,) to identify cutaneous nerve fibers. Western blots of dorsal root ganglion protein immunostained with PGP9.5 antibody showed a band of 27kDa, serving as a positive control (Doran et al., 1983). Sections were blocked with donkey serum followed by overnight incubation with primary antibodies, rinsing in PBS with 3% triton for 30 minutes, and incubation for one hour with secondary antibodies (Cy3

Donkey anti-Goat and Alexa 488 Donkey anti-Rabbit; Jackson ImmunoResearch Laboratories, Inc. West Grove, PA). Sections were rinsed in PBS with 3% triton and mounted with Fluoromount G.

Confocal Imaging

Images were taken with 60X or 100X oil immersion objectives on an Eclipse 90i microscope equipped with a Nikon C1si confocal system and a D-Eclipse camera (Melville, NY). 2-D images were created using maximum projection on volume renderings of Z-stacks. To create 3-dimensional (3-D) images, image acquisition and volume rendering of Z-stacks were performed using Nikon NIS-elements Advance Research 3.10 software (Melville, NY).

Stereological Analysis of LCs

For each animal, three sample fields ($0.148\text{mm}^2/\text{field}$) per section were obtained with a 20X objective using a Photometrics Cool SNAP-EZ Camera (Roper Scientific Inc., Tucson, AZ) and Nikon Eclipse TE300 inverted microscope, and analyzed (MetaMorph, Molecular Devices, Downingtown, PA). Epidermal area within the sample field was estimated by tracing around the stratum granulosum and basal cell layers while excluding the stratum corneum. Numbers of cells -positive for langerin immunoreactivity (+) were counted. The contribution of langerin+ cells to the overall epidermal sample area was obtained by superimposing a stereological grid ($13.23\mu\text{m}$ intersections), counting numbers of intersects overlying LCs, and multiplying by the grid square area corresponding to each intersection ($175\mu\text{m}^2$), using approaches similar to previous studies (Zoubina et al., 1998; Blacklock et al., 2004; Blacklock and Smith, 2004). The apparent percentage of epidermal area occupied by langerin+ cells was also calculated by dividing total langerin area by epidermal area and then multiplying by 100. To obtain an index of overall LC size, total langerin+ area was divided by the number of langerin+ cells.

Quantitation of IENFs

A method originally defined by Kennedy et al. (Kennedy et al., 2005) and adopted by European Federation of Neurological Societies (EFNS) (Lauria et al., 2005a) was used to quantify epithelial innervation. Individual IENFs immunostained for PGP9.5 that cross the dermal-epidermal junction were counted while excluding any secondary branching. Counts were divided by the length of the epidermis and expressed as IENF/mm. Individual IENFs were visually counted at three regions along the length (distal, middle, and proximal) of each footpad section with a 40X objective and 10X eyepiece on a Nikon Eclipse TE300 inverted microscope (Nikon Corp., Tokyo, Japan). Each region spanned 0.42mm. To determine epidermal length, the basement membrane was measured. For each region, IENF counts were divided by epidermal length to obtain PGP9.5+ IENF/mm. We also repeated these counts in samples double stained for langerin, in which PGP9.5+ projections co-labeled with langerin were excluded from counts. These data are expressed as PGP9.5+/langerin- IENF/mm.

Statistical Analysis

Weight and blood glucose levels were analyzed with two way repeated measures analysis of variance and Student-Newman-Keuls post-hoc analysis. Differences in epidermal LC density, size, and percent epidermal area were compared by t-test or two way analysis of variance and Student-Newman-Keuls post-hoc analysis. IENF/mm data were compared by t-test with Bonferroni correction. The effect of age on nerve density was analyzed with two way analysis of variance. Differences were considered significant if p value was ≤ 0.05 . Data are expressed as Mean \pm Standard Error Mean (SEM).

Results

Blood glucose and body weight

To confirm diabetic status at 4 and 16 weeks following STZ injections, blood glucose levels were measured (Table 1). Vehicle-injected rats remained in the euglycemic range, while glucose levels of STZ-treated rats at 4 and 16 weeks were significantly greater than their age-matched controls ($p < 0.001$ and $p < 0.001$, respectively). Moreover, glucose levels for STZ-treated rats at 16 weeks were higher than levels at 4 weeks post-STZ ($p < 0.001$). Age did not affect body weight within vehicle- and STZ-treated groups (Table 1). However, body weight was significantly reduced after 4 and 16 weeks of diabetes ($p < 0.001$ and $p < 0.001$).

Langerin immunostaining in euglycemic rats at 3 months of age

Immunostained footpad sections of 3-month-old rat 4 weeks following vehicle injection revealed a langerin+ epidermal cell population. Langerin+ cells were primarily found within the stratum spinosum, granulosum and basal layers (Fig. 1a), but seen occasionally in the stratum corneum and below the basement membrane (not shown). Langerin+ cells were stellate or spindle-shaped with extensive processes, sometimes stretching across all epidermal layers except the stratum corneum. Langerin+ appeared to be intra-cytoplasmic and punctuate, localizing mainly to the soma with less accumulation in distal-most processes (Fig. 1a, See Electronic Supplementary Movie 1, S1).

Langerin immunostaining in euglycemic rats at 6 months of age

LCs appeared to be increased in non-diabetic rats at 6 months relative to 3 months (Fig. 1b). Quantitative analysis showed a 75% increase in LC density in non-diabetic animals when compared to 3 month old non-diabetic animals, ($p < 0.001$, Fig. 1e). Moreover, at 6 months of

age, LCs had longer processes and slightly larger somas when compared to three month old animals (Fig. 1b). LC size was 38% greater at 6 months as compared to rats at 3 month of age ($p<0.001$, Fig. 1f). Overall, the percentage of epidermal area occupied by LCs increased by 141% between 3 and 6 months of age ($p<0.001$, Fig. 1g).

Langerin immunostaining in hyperglycemic rats at 3 months of age

Rats at 3 months of age that received a single i.p. injection of STZ 4 weeks earlier appeared to have fewer LCs when compared to 3 month old euglycemic rats (Fig. 1c), and quantitative analysis confirmed a 17% decrease ($p=0.032$, Fig. 1e). However, 4 weeks of diabetes did not alter either the average size of LCs (Fig. 1f) or the percentage of epidermal area occupied by LCs (Fig. 1g).

Langerin immunostaining in hyperglycemic rats at 6 months of age

Rats aged 6 months rendered diabetic for 16 weeks displayed numerous small LCs in their epidermis (Fig. 1d). Relative to younger diabetic rats, there was a 76% increase in LCs in 6-month-old diabetic rats ($p<0.001$, Fig. 1e). However, the magnitude of the age-related increase that occurred in non-diabetic rats was attenuated by diabetes ($p=0.041$, Fig. 1e). In addition, LC size showed no age-related increase between 3 and 6 months in diabetic animals (Fig. 1f). When compared to aged controls, LC sized decreased by 37% after 16 weeks of diabetes ($p<0.001$, Fig. 1f). The overall percentage of epidermal area occupied by LCs increased by 61% between 3 and 6 months, but remained markedly lower than that of euglycemic rats at 6 months ($p<0.001$, Fig. 1g).

PGP 9.5 immunostaining of Langerhans cells after 4 weeks of diabetes

Footpad sections from three-month-old non-diabetic and diabetic rats one month after inducing diabetes showed PGP9.5+ within the cytoplasm of stellate and spindle-shaped cells within non-keratinized epidermis (Fig. 2a and b, Electronic Supplementary Movie, S2). These cells frequently displayed long processes oriented perpendicularly to the epidermal longitudinal axis (Fig. 2a, Electronic Supplementary Movie, S2).

Co-stained sections were examined to define the contributions of LCs to PGP9.5+ structures. Langerin revealed populations of epidermal cells as described above (Fig. 2c and d). Double-staining showed co-localization of langerin in PGP9.5+ stellate-shaped cells (Fig. 2e and f, Electronic Supplementary Movie, S2). PGP9.5 expression was uniform within the cell body but was often patchy within LC processes. In addition, PGP9.5 was occasionally located only on profiles that appeared to contact langerin+ LC somata. All cells with PGP9.5+ also contained langerin+. However, some langerin+ LCs within the epidermis (Fig. 2e and f) and occasionally within the immediate sub-epidermal layer (not shown) lacked PGP9.5+. The expression patterns of langerin and PGP9.5 in LCs did not appear to be altered as a result of 4 weeks of diabetes (Fig. 2.a-f).

PGP 9.5 immunostaining of Langerhans cells after 16 weeks of diabetes

Epidermal sections of 6-month old rats that received streptozotocin or vehicle injection 4 months earlier showed langerin and PGP9.5 immunostaining similar to that of rats at 3 months of age. Co-localization of PGP9.5 and langerin within LCs also appeared comparable and did not appear to be affected by diabetes (Fig. 3a-f). The increased langerin+ LC numbers associated with aging was accompanied by a proportionate increase in numbers of PGP9.5+ cells in euglycemic and hyperglycemic rats. Similarly, the increased size of LCs in the non-

diabetic rats appeared to be accompanied by proportionate increases in PGP9.5 immunoreactivity, so that there were no obvious changes in LC phenotype.

Intraepidermal nerve fibers

Footpad sections double-stained for PGP9.5 and langerin were examined to determine how age and diabetes affect IENFs and their relationship to LCs. PGP9.5 immunostaining revealed large nerve bundles running parallel to the basement membrane. Smaller branches emanated from the bundles and penetrated the epidermis perpendicularly (Fig. 2a and b).

To examine the spatial arrangement of PGP9.5+ axons and langerin+ LCs, 3-D reconstruction of confocal images from double-stained footpad sections was performed (Electronic Supplementary Movie, S2). Confocal images revealed PGP9.5+ fibers that could be traced to subepidermal nerve bundles frequently showed intimate associations with langerin+ LCs; nerve fibers were also frequently intermingled between the fine processes of the LCs (Figs. 2 e and f, 3e and f, Electronic Supplementary Movie, S2). In fact, in PGP9.5 immunostained specimens, it was often impossible to distinguish with certainty which 'fibers' were axons and which were LC projections.

To determine whether LC projections contribute to counting errors that may occur when quantifying epidermal innervation, we performed IENF counts using images immunostained for PGP9.5 and langerin. Sections co-stained for langerin revealed LC extensions could be discerned more readily. PGP9.5+ LC processes contributed 15-19% of overall IENF counts for non-diabetic rats at both 3 (Fig. 2g) and 6 months of age (Fig. 3g), but achieving statistical significance only at 3 months ($p=0.024$, Fig. 2g). LC contributions to counts in diabetic groups were more modest and not statistically significant (Fig. 2g and 3g).

In non-diabetic rats we detected a decrease in numbers of both corrected (i.e., LC processes excluded, $p=0.005$) and uncorrected ($p<0.001$) IENF counts between 3 and 6 months of age (Fig. 2g and 3g). There were no differences in corrected and uncorrected IENF counts between 3 and 6 month old diabetic rats. Irrespective of whether IENF counts were corrected for LC contributions, there was no significant difference between diabetic and non-diabetic rats at 4 and 16 weeks after induction (Figs. 2g and 3g).

Discussion

Cutaneous infections are the primary cause of lower limb amputation among diabetic patients. Understanding the effect of diabetes on APCs, a primary defense mechanism against external pathogen invasion, is therefore critical. While a number of proteins have been used to identify LCs, langerin is perhaps the most selective marker, at least in mice and humans (Valladeau et al., 1999; Stoitzner et al., 2003). Although rats are frequently used in research on diabetes, aging and neuro-immune interactions, to date descriptions of langerin expression in the rat are limited (Meyer et al., 2010). Concordant with reports in other species, our immunostaining studies show langerin⁺ is present throughout the soma and processes of all identified LCs, confirming langerin is a useful LC marker in rat.

An effect of aging on immune status and immune cell function is not unexpected given an animal's cumulative exposure to antigens in the environment. Accordingly, we found that LC density increased between 3 and 6 months in non-diabetic rats, implying greater LC proliferation (i.e. more cell migration or replication) in older rats. LC size also appeared to increase with age. Previous studies proposed that LC size is an indicator of functional maturity in LCs (Larsen et al., 1990; Nishibu et al., 2006). Our findings therefore suggest that the proportion of functionally

mature LCs is greater in the older rats. Collectively, the increase in LC size and numbers resulted in an overall increase in the relative contribution of these cells to the foot pad skin, implying that LCs may be able to survey a greater volume of foot epidermis in older animals.

Age-related changes in epidermal LC composition may vary as a function of species and location. The rat foot pad represents the main point of physical contact with the external environment and therefore is constantly exposed to potential antigens. In contrast, studies in mice and humans suggest that other sites may not undergo similar increases; for example, skin samples from ear and buttocks showed evidence of decreased LC composition with age (Bhushan et al., 2002; Cumberbatch et al., 2002; Agrawal et al., 2007). Varied findings may be due to differences in species (Breathnach, 1991), skin type (e.g., glabrous vs. hairy), location (Berman et al., 1983; Thomas et al., 1984), and differences in age-related LC changes may not be surprising. Nonetheless, the foot is of particular clinical importance because it is frequently affected by cutaneous ulceration and infection in diabetic patients, often resulting in amputation. Hence an understanding of changes in LCs in the hind paw is of particular clinical relevance.

Relative to control subjects, rats with STZ-induced diabetes had reduced hind paw LC density at 4 weeks post-treatment. However, neither LC size nor the percentage of epidermal area occupied by LCs was statistically different from control values. These results suggest that in the early stages, diabetes selectively reduces LC numbers, presumably by reducing the proliferation of these cells. Despite this reduction, the overall contribution of LCs to foot pad cutaneous composition remained relatively normal. Although functional studies are necessary to determine whether a normal immune response is retained, these findings imply that area of cutaneous tissue surveyed by LCs remains relatively normal in early STZ-induced diabetes.

In contrast, rats that endured 16 weeks of hyperglycemia showed decreases in LC density, size and the percentage of epidermal area occupied by LCs. This suggests that a longer duration of hyperglycemia may impair both proliferation and maturation. These findings are in agreement with those of Zeigler and Standl who found a reduction in LCs in early Type I diabetes in humans (Ziegler and Standl, 1988), but differ from a report of increased LCs in the STZ diabetic rat (Lauria et al., 2005b). As noted above, differences may reflect regional variations, but may also represent differences in quantitative methods (correcting for length only vs. epidermal volume), and in LC markers (langerin vs. PGP9.5, which does not label all LCs). In any event, our study shows diabetes can have a negative effect on LC proliferation and maturation, leading to reduced epidermal antigen surveillance.

As in euglycemic rats, the percentage of epidermal area occupied by LCs in diabetic rats at 6 months of age was increased relative to that at 3 months, although the increase was attenuated in diabetic animals. This was due to combined effects on LC proliferation and maturation, as diabetes diminished both cell density and size. Thus, duration of diabetes appears to affect LC dynamics negatively. It is unclear whether age of onset is also a factor. In any event, the effects of diabetes were certainly more pronounced in aged rats.

Because previous reports have suggested that changes in epidermal innervation can alter LC density, it was important to determine if innervation was affected in our STZ-diabetic rats. However, because LCs express PGP 9.5 and are often intimately associated with axons, we were concerned that fine PGP 9.5+ processes from LCs could contribute to IENF counts. We therefore used dual immunostaining to more fully investigate the relationship between axons and LCs. Most LCs expressed both langerin and PGP 9.5, but a significant proportion of LCs expressed langerin but not PGP 9.5, with the incidence of these cells increasing towards the apical portion of the dermal papillary layer. Variation in PGP+ has been reported previously, but

only in association with phenotypic changes occurring with loss of cutaneous innervation (Hsieh et al., 1996). These findings indicate that while a number of LCs do not express PGP 9.5, most LCs do express this neuroendocrine marker.

‘Axon’ counts in material double-stained for langerin revealed that PGP 9.5+ LC processes may be a potential source of error. While this occurred in all specimens, the contribution was greatest within non-diabetic animals. We attribute this to the findings that diabetes reduces the number, size and complexity of LCs. These results show that PGP9.5+ within LC processes can be a source of error in IENF counts if they are not excluded by co-staining with a more selective LC marker such as langerin.

When we compared IENF density between 3 and 6 months in non-diabetic rats, we found a significant decrease in IENFs when counts were either corrected or not corrected for contributions PGP 9.5+ LC processes. These findings agree with those of others who also report a decrease in innervation with age (Goransson et al., 2004; Lauria et al., 2010). It appears that cutaneous density decreases in rats as a function of age.

In contrast, there was no clear age-related decline in IENF density in diabetic rats. At 4 weeks post-treatment, axon numbers did not differ significantly from controls irrespective of whether or not we corrected for LC contribution. This is in accord with a prior report failing to detect axon reductions after 4 weeks of diabetes (Karanth et al., 1990). Similarly, IENF counts at 16 weeks post-treatment were not significantly different from those at 4 weeks. This finding was somewhat unexpected as several other studies have reported reduced numbers of cutaneous fibers in rats at 12 weeks or later post-STZ (Lauria et al., 2005b; Toth et al., 2006; Leonelli et al., 2007; Roglio et al., 2007; Jin et al., 2009). Because our rats showed all the classical signs of insulin-deficient diabetes including hyperglycemia, weight loss, polydipsia and

polyuria, the difference is unlikely to be attributable to ineffective STZ treatment. Other factors that may contribute to differences in observed changes in IENFs include age at which diabetes is induced, method of induction, and sampling and quantitation approaches. In any case, there was no significant loss of cutaneous nerve fibers in our diabetic rats, indicating that LC changes in diabetes are not likely to be secondary to outright nerve degeneration.

It is unclear how diabetes alters LC dynamics. One mechanism may involve altered production of granulocyte-macrophage colony-stimulating factor (GM-CSF). GM-CSF is necessary for LC proliferation and maturation (Heufler et al., 1988; Kingston et al., 2009). Diabetes reduces GM-CSF production in wounds by 50% (Fang et al., 2010), and delayed wound healing in diabetes is reversed by GM-CSF administration (Fang et al., 2010). Therefore, diminutions in GM-CSF may impair recruitment and maturation of LCs in diabetes.

Collectively, this study shows that LCs are strongly influenced by aging and diabetes. Given that both aging and diabetes can be predisposing factors in development of cutaneous infections and ulcerations, our findings suggest that effects on dendritic cells may well play a role in the development of these pathologies.

Tables

Table 1 Blood Glucose Levels and Body Weight at End of Experiment

	Glucose (mg/dL)	Weight (grams)
<u>3 months</u>		
Non-diabetic	91 ± 5	282 ± 8
Diabetes, 4 wks	408 ± 34*	233 ± 10*
<u>6 months</u>		
Non-diabetic	120 ± 9	306 ± 4
Diabetes, 16 wks	529 ± 22*	219 ± 11*

Table 1 Blood Glucose Levels and Body Weight at End of Experiment

Blood glucose levels were significantly higher than controls after 4 and 16 weeks of diabetes.

Glucose levels were significantly greater after 16 weeks of diabetes when compared to 4 weeks of diabetes. Body weight significantly decreased after four and 16 weeks of diabetes. Data is presented as mean \pm SEM. * $p < 0.001$

Figures

Figure 1

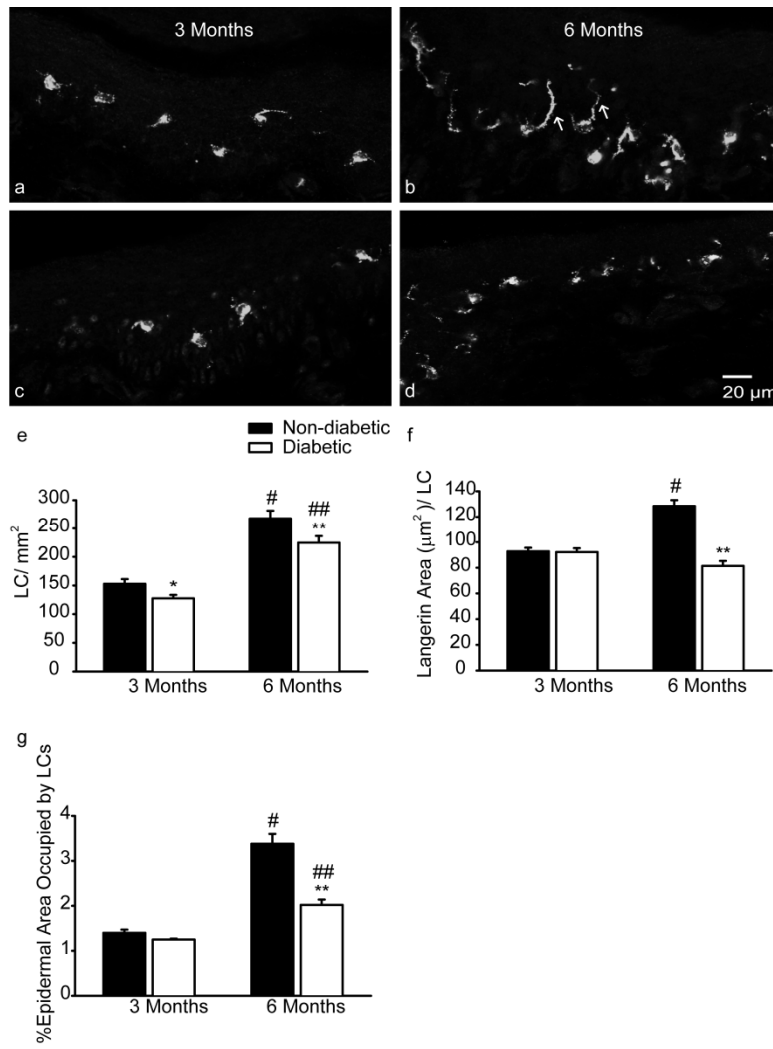


Figure 1 Quantitative Analysis of Langerin+ LCs in the Rat Footpad

The effect of age on LCs was quantified by comparing three month old non-diabetic (a) and diabetic animals (c) to six month old non-diabetic (b) and diabetic animals (d), respectively. The effect of STZ treatment on LCs was quantified by comparing non-diabetic animals at four and 16 weeks post-treatment (a and b, respectively) to diabetic animals at four and 16 weeks post-treatment (c and d, respectively). White arrows reveal longer processes on LCs in six month old non-diabetic animals. Bar graphs reflect LC density (e), size (f), and the % of epidermal area occupied by LCs (g) at 3 (4 weeks post-STZ) and 6 months (16 weeks post-STZ) of age. *: $p=0.032$ when compared to 4wk non-diabetic animals, #: $p<0.001$ when compared to 4 week non-diabetic animals, ##: $p<0.001$ when compared to 4 week diabetic animals, **: $p<0.001$ when compared to 16 week non-diabetic animals

Figure 2

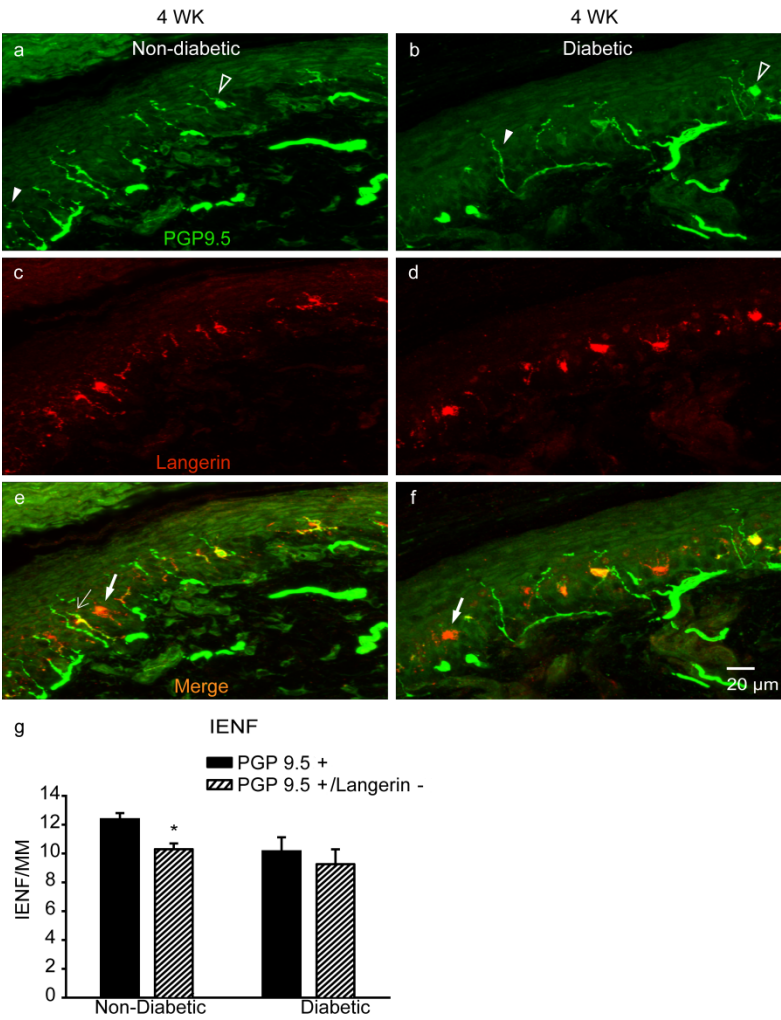


Figure 2 LC contribution to IENF values at 4 Weeks of diabetes

Footpad sections were double-stained with a PGP9.5 (a and b) and langerin (c and d) antibody to identify IENFs and LCs, respectively. PGP9.5 and langerin images were merged (e and f) to display LC contribution to PGP9.5(+) IENFs in non-diabetic (a, c, and e) and diabetic animals (b, d, and f) at four weeks post-treatment. Open arrowhead designates PGP9.5+ LCs. Filled arrowheads mark an IENF that branches from dermal nerve bundle and enters epidermis. Thin arrow indicates PGP9.5+/Langerin+ that would be considered a PGP9.5+ IENF if langerin immunoreactivity was not considered. Closed arrow reflects PGP9.5-/Langerin+ LCs. Before langerin immunoreactivity was considered, IENF density was quantified (solid bars). After PGP9.5+ profiles that express langerin were excluded, PGP9.5+/langerin- IENFs were calculated (dashed bars). * $p=0.024$ as compared to non-diabetic PGP9.5+ IENF density

Figure 3

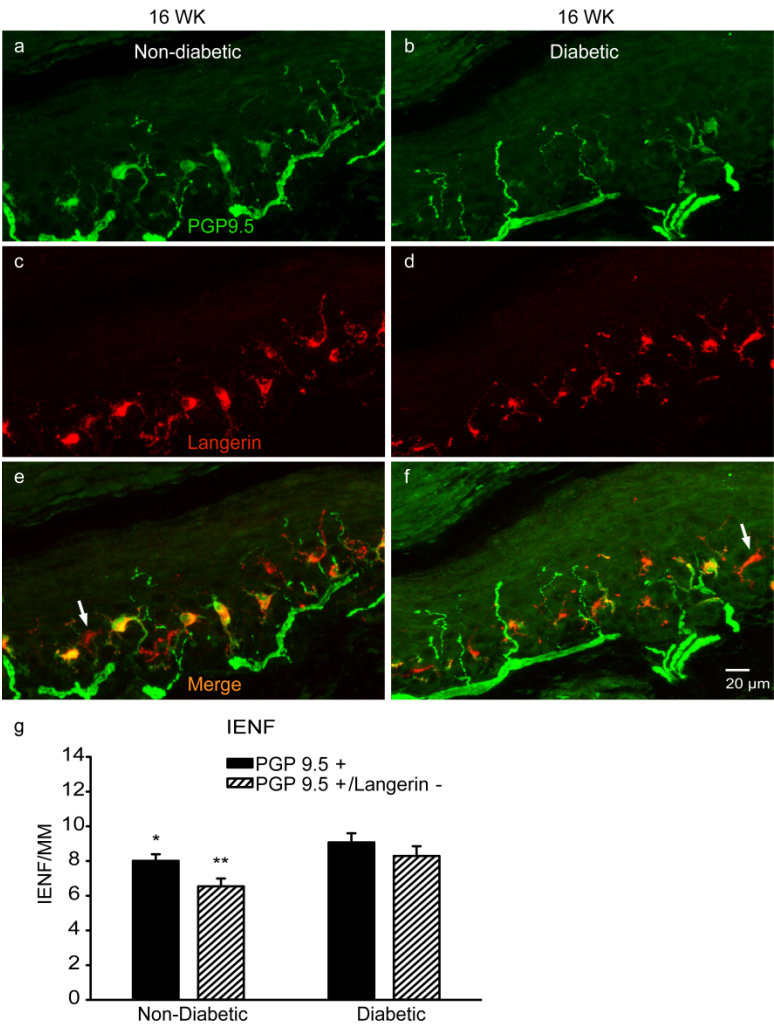


Figure 3 LC Contribution to IENF Values at 16 Weeks of diabetes

Footpad sections from non-diabetic animals (a, c, and d) and diabetic animals (b, d, and f) were double-stained with PGP9.5 (a and b) and langerin (c and d) antibodies. Merged images (e and f) reveal LC contribution to IENF density by means of PGP9.5 expression. Closed arrow reflects PGP9.5-/Langerin+ LCs. Before langerin immunoreactivity was considered, IENF density was quantified (solid bars). After PGP9.5+ profiles that express langerin were excluded, PGP9.5+/langerin- IENFs were calculated (dashed bars). * $p < 0.001$ when compared to PGP9.5+ IENF density for 3 month old non-diabetic rats and ** $p = 0.005$ when compared to PGP9.5+/langerin- IENF density for 3 month old non-diabetic rats

Electronic Material Legend

Electronic Supplementary Material Movie 1 (S1). Langerin Expression within LCs in the Non-Diabetic Footpad at Four Weeks post-treatment. 3-D reconstruction of confocal images taken with a 100X objective from a footpad section double-stained with a langerin (red) antibody shows langerin is expressed within LCs.

Electronic Supplementary Material Movie 2 (S2). Langerin and PGP9.5 Immunoreactivity within LCs in the Non-Diabetic Footpad at 16 Weeks Post-Treatment. Footpad section was immunostained with a PGP9.5 (green) langerin (red) antibody. 3-D reconstruction of confocal images taken with a 100X objective revealed PGP9.5 and langerin immunoreactivity in the soma and processes of LCs. Moreover, 3-D imaging reveals LCs are intricately intermingled between IENFs, and their PGP9.5+ processes often stretch towards the basement membrane.

Chapter 4: General Discussion

It is widely accepted that a bi-directional crosstalk exists between immune cells and nerves. The nervous system is capable of modulating immune cells through neuropeptide expression and this includes LCs. Data suggests many immune cells may be capable of regulating nerve fibers, possibly through pro-inflammatory cytokines and neurotrophic factor expression. However, it is unclear whether LCs influence nerves. As a result, the objective of this study was to determine whether LCs are necessary for maintaining IENFs and if so, is this newly discovered role altered by age or diabetes. If LCs are required for proper epidermal innervation, LCs may become a new target for developing drugs to achieve and maintain epidermal innervation.

Langerhans cells are necessary for maintaining intraepidermal nerve fibers

With the use of the Lang-DTR mouse model, this study showed LCs provide a substantial amount of support for IENFs. Results revealed LCs help maintain peptidergic IENFs in 1 month old mice. To determine whether LCs are influencing IENFs through neurotrophic factors, gene expression for common neurotrophic factors were examined. Results from 1 month old mice showed LCs control roughly 75% and 90% of NGF and GDNF gene expression, respectively. This implies that LCs may be regulating peptidergic IENFs by manipulating NGF and GDNF expression. When cutaneous sensitivity was examined, data showed LC depletion increased mechanical sensitivity which was associated with peptidergic IENF loss. Since C fibers can detect mechanical stimuli (Teliban et al., 2011) and peptidergic IENFs are a subtype of C fibers, these data imply LCs provide trophic support for peptidergic C fibers that are essential for properly recognizing mechanical stimuli. Our data also supports previous studies that report allodynia can be associated with C fiber loss (Xiao et al., 2009) and neuropathy (Hovaguimian and Gibbons, 2011).

Research shows aging can be associated with a reduction in immune cells and innervation in the skin (Belsito et al., 1989; Goransson et al., 2004). Therefore, it is reasonable to hypothesize that the newly discovered relationship between LCs and IENFs may be altered in older mice. As a result, LCs and IENFs were examined in older mice in addition to whether age altered LCs role in IENF maintenance. LCs and IENFs were reduced with aging. Since the first study showed LCs are necessary for maintaining IENFs, it is possible that a reduction in IENFs may be due to a reduction in LC. A reduction in LC-IENF interaction may help explain why aging is linked to increased susceptibility to infection and delayed wound healing (Pittman, 2007; Ongradi et al., 2009; Prakash and Davis, 2010).

Quantification of IENF subtypes revealed the subtype influenced changed from peptidergic to non-peptidergic with age. Data from 1 month old mice showed IENF degeneration was associated with reduced neurotrophin expression; this was not the case for older mice. The lack of change in neurotrophic factor expression indicates that LCs do not help maintain IENFs through neurotrophic factor expression in older mice and that another pathway is used. Although more studies are needed to determine how LCs provide trophic support in older mice, these data provide a new function for LCs.

Collectively, the first study reveals LCs play a fundamental role in maintaining epidermal innervation. In addition, inconsistent changes in neurotrophic factor expression with age suggest the process by which LCs provided support involves multiple pathways. Despite this conundrum, our study provides evidence that the relationship between LCs and epidermal nerve fibers is a two-way street and that future studies are needed to determine the mechanism(s) LCs employ to help maintain IENFs in older mice. Information from this study suggests that a reduction in LC status may be an early indicator of small fiber neuropathy associated with

Guillain-Barré syndrome and chronic inflammatory demyelinating polyneuropathy, diabetes, HIV/AIDS, and alcoholism.

Diabetes reduces Langerhans cells but not intraepidermal nerve fibers

DM is often associated with delayed wound healing and chronic severe cutaneous infections. Although many factors such as a suppressed immune system and small fiber neuropathy are known to contribute to the severity of these complications, little is known about DM's effect on LCs and their relationship with IENFs in the skin. Since results showed LCs provide neurotrophic support, it is possible that a reduction in LCs caused by diabetes could lead to a reduction in IENFs. The purpose of the second study was to examine whether STZ-induced diabetes in the rat causes a reduction in LCs which could lead to IENF degeneration.

LCs and IENFs were examined after 4 and 16 weeks of STZ-induced diabetes. Due to the fact that rats were three months older after 16 weeks of STZ-induced diabetes and that age can influence LCs and IENFs, it was necessary to examine age as a modulator of LCs and IENFs. LC proliferation and maturation increased in older rats. This was different from what was observed in mice. This may be because mice were allowed to age more (~9 months) than rats (3 months). Therefore, it may have been too early to observe aging-induced LC reduction in the rat. Also, it is unclear whether results are due to a difference in species.

IENF density decreased with aging rats. Since we showed LCs are necessary for IENF maintenance, was reasonable to conclude that a reduction in IENFs was due to a reduction in LCs. However, this hypothesis does not hold true for the rats in this study. In this case, IENF loss was associated with an increase in LC status. This suggests that the relationship between

LCs and IENFs in the rat may not be similar to that of the mouse. However, since aged mice were roughly 6 months older than the rats, it is not reasonable to disregard the potential role LCs play in maintaining IENFs in rat skin. For the most part, our data agrees with studies that report a decrease in LCs and IENFs with aging (Bhushan et al., 2002; Cumberbatch et al., 2002; Goransson et al., 2004; Lauria et al., 2010).

Data showed STZ-induced diabetes did in fact reduce LC proliferation and maturation. Moreover, this effect was more apparent with a longer duration of hyperglycemia. Analysis of IENFs revealed IENF status did not change with STZ-induced diabetes. These data imply that LCs are more sensitive to hyperglycemia than IENFs after 16 weeks of streptozotocin-induced diabetes in the rat. Although small fiber neuropathy was not observed, we cannot rule out that neuropathy could occur after a longer period of hyperglycemia and that a reduction in LCs occurs prior to IENF loss. Previous studies report changes in IENFs can modulate LC density. Since diabetes did not alter IENFs, this data also show that a reduction in LCs is more likely a direct effect of diabetes than changes in IENFs. Altogether, this data indicate a reduction in LCs by diabetes may lead to a reduction in immunosurveillance. Decreased immunosurveillance could potentially lead to increased susceptibility to severe infections.

Conclusion

These data show LCs play an essential role in maintaining IENF density in mice. As animals age, the fiber subtype influenced by LCs switches from peptidergic to non-peptidergic IENFs. LCs provide support for IENFs through NGF and GDNF in young mice. However, the mechanism by which LCs support IENFs is altered with age which suggests this process includes multiple pathways. In addition, LCs help maintain proper mechanical sensitivity in the

skin through regulating peptidergic C fibers. Similar to previous studies, aging is associated with a reduction in LCs and IENFs in the mouse. Despite conflicting results with LCs in the rat, a reduction in IENFs in the mouse may be due to a reduction in LCs. In a STZ-induced diabetic environment, LC reduction precedes IENF degeneration. This may imply that after a certain amount of LC reduction, IENF degeneration occurs in the rat. Altogether, this suggests that changes in LC status may be an early indicator and contributor to abnormal innervation and sensitivity associated with atopic dermatitis, psoriasis, eczema, and various forms of small fiber neuropathy.

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